#6

RPR File No: ST95021-US

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re the Application of:	Bracco et al.	Group Art Unit:
Serial No.:	08/930,480	Examiner:
	U.S. National Stage of PCT/FR96/00477	
Filed:	September 29, 1997	
For:	Conditional Expression System	

To: The Honorable Commissioner of Patents and Trademarks
Box PCT
Washington, D.C. 20231
Attn: EO/US

CERTIFICATE OF MAILING (37 CFR 1.10)

GB84071613X US
"Express Mail" Mailing Number

January 21, 1998
Date of Deposit

I hereby certify that this paper (along with any referred to as being attached or enclosed) is being deposited with the United States Postal Service "Express Mail Post Office to Addressee" service under 37 CFR 1.10 on the date indicated above and is addressed to Commissioner of Patents and Trademarks, Box PCT, Washington, DC 20231, Attn. EO/US.

Paula L. Dickey
(Type or print name of person mailing paper)

Paula L. Dickey
(Signature of person mailing paper)

SUBMISSION OF DECLARATION OF THE INVENTORS UNDER 37 CFR § 1.63

In response to the "Notification of Missing Requirements Under 35 U.S.C. 371" mailed November 26, 1997 (a copy of which is attached), Applicants submit herewith the executed Declaration and Power of Attorney for the above-identified patent application.

Please charge Deposit Account No. 18-1982 in the amount of $130.00 to cover the surcharge for providing the Declaration later than 30 months from the priority date. This sheet is provided in triplicate.

Applicants submit herewith a Petition for a one (1) month Extension of Time Under 37 CFR § 1.36(a), extending the period of response to the Notification to January 26, 1998.

Rhône-Poulenc Rorer Inc.
P.O. Box 5093, Mail Stop 3C43
Collegeville, PA 19426-0997
Telephone: (610) 454-3839
Facsimile: (610) 454-3808

Dated: 1/20/98

Respectfully submitted,

Paul F. Fehlner

Paul F. Fehlner, Ph.D.
Attorney for Applicants
Registration No. 35,135

Rec'd PCT/PTO 29 SEP 1997

FORM PTO-1390 (REV. 5-93) U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE	ATTORNEY'S DOCKET NUMBER
TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371	ST95021-US U.S. APPLICATION NO. (If known, see 37 CFR 1.5) 08/930480

INTERNATIONAL APPLICATION NO.	INTERNATIONAL FILING DATE	PRIORITY DATE CLAIMED
PCT/FR96/00477	29 March 1996	31 March 1995

TITLE OF INVENTION

CONDITIONAL EXPRESSION SYSTEM

APPLICANT(S) FOR DO/EO/US

Laurent BRACCO, Fabien SCHWEIGHOFFER and Bruno TOCQUE

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. [X] This is a FIRST submission of items concerning a filing under 35 U.S.C. 371.
2. [ ] This is a SECOND or SUBSEQUENT submission of items concerning a filing under 35 U.S.C. 371.
3. [X] This is an express request to begin national examination procedures (35 U.S.C. 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39(1).
4. [X] A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.
5. [X] A copy of the International Application as filed (35 U.S.C. 371(c)(2))
   a. [ ] is transmitted herewith (required only if not transmitted by the International Bureau).
   b. [X] has been transmitted by the International Bureau.
   c. [ ] is not required, as the application was filed in the United States Receiving Office (RO/US)
6. [X] A translation of the International Application into English (35 U.S.C. 371(c)(2)).
7. [X] Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3))
   a. [ ] are transmitted herewith (required only if not transmitted by the International Bureau).
   b. [ ] have been transmitted by the International Bureau.
   c [ ] have not been made; however, the time limit for making such amendments has NOT expired.
   d. [X] have not been made and will not be made.
8. [ ] A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
9. [X] An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)), and Power of Attorney, unsigned.
10. [ ] A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).

Items 11. to 16. below concern other document(s) or information included:

11. [ ] An information Disclosure Statement under 37 CFR 1.97 and 1.98.
12. [ ] An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
13. [X] A FIRST preliminary amendment.
    [ ] A SECOND or SUBSEQUENT preliminary amendment.
14. [ ] A substitute specification.
15. [ ] A change of power of attorney and/or address letter.
16. [X] Other items or information: Submission of Computer Readable Sequence Listing; Paper Copy Sequence Listing; and CRF (disk) copy Sequence Listing.

CERTIFICATION UNDER 37 CFR 1.10

GB840711234 US	29 September 1997
"Express Mail" Mailing Number	Date of Deposit

I hereby certify that this paper (along with any referred to as being attached or enclosed) is being deposited with the United States Postal Service "Express Mail Post Office to Addressee" service under 37 CFR 1.10 on the date indicated above and is addressed to Box PCT, Assistant Commissioner for Patents, Washington, D.C. 20231, Attn. EO/US

Paula L. Dickey	Paula L. Dickey
(Type or print name of person mailing paper)	(Signature of person mailing paper)

U.S. APPLICATION NO. (If Known, see C.F.R. 1 5)	INTERNATIONAL APPLICATION NO.	ATTORNEY'S DOCKET NUMBER
	PCT/FR96/00477	ST95021-US

17. [X] The following fees are submitted:

Basic National Fee (37 CFR 1.492(a)(1)-(5):
Search Report has been prepared by the EPO or JPO.....$ 910 00

International preliminary examination fee paid to USPTO (37 CFR 1.482) .....$ 700.00

No international preliminary examination fee paid to USPTO (37 CFR 1.482)
but international search fee paid to USPTO (37 CFR 1.445(a)(2))..$ 770.00

Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid USPTO.....$1040.00

International preliminary examination fee paid to USPTO (37 CFR 1.482) and all claims satisfied provisions of PCT Article 33(2)-(4).....$ 96.00

	CALCULATIONS	PTO use only
ENTER APPROPRIATE BASIC FEE AMOUNT =	$ 910.00	
Surcharge of $130.00 for furnishing the oath or declaration later than [ ] 20 [ ] 30 months from the earliest claimed priority date (37 CFR 1.492(e)).	$	

Claims	Number Filed	Number Extra	Rate		
Total Claims	20 -20 =	0	X $ 22.00	$	
Independent Claims	1 - 3 =	0	X $ 80.00	$	
Multiple dependent claim(s) (if applicable)			+ $260.00	$	
TOTAL OF ABOVE CALCULATIONS =				$ 910.00	
Reduction by 1/2 for filing by small entity, if applicable. Verified Small Entity statement must also be filed. (Note 37 CFR 1.9, 1.27, 1.28).				$	
SUBTOTAL =				$ 910.00	
Processing fee of $130.00 for furnishing the English translation later the [ ] 20 [ ] 30 months from the earliest claimed priority date (37 CFR 1.492(f)). +				$	
TOTAL NATIONAL FEE =				$ 910.00	
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). $40.00 per property +				$	
TOTAL FEES ENCLOSED =				$ 910.00	
				Amount to be refunded	$
				charged	$

a. [ ] A check in the amount of $_____ to cover the above fee is enclosed.

b. [X] Please charge my Deposit Account No. 18-1982 in the amount of $910.00 to cover the above fees. A duplicate copy of this sheet is enclosed.

c. [X] The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 18-1982. A duplicate copy of this sheet is enclosed.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1 137(a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO:
Paul F. Fehlner, Esquire
Rhone-Poulenc Rorer Inc.
Legal-Patents, #3C43
P.O. Box 5093
Collegeville, PA 19426-0997
Telephone: (610) 454-3839
Facsimile: (610) 454-3808

Signature Paul F Fehlner

Paul F. Fehlner
Name

35,135
Registration Number

Date Sept. 27, 1997

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re the Application of:	Bracco et al.	Group Art Unit:
Serial No.:	To Be Assigned	Examiner:
	U.S. National Stage of PCT/FR96/00477	
Filed:	Concurrently Herewith	
For:	Conditional Expression System	

To: The Honorable Commissioner of Patents and Trademarks
Washington, D.C. 20231

CERTIFICATE OF MAILING (37 CFR § 1.10)

GB840711234US
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September 29, 1997
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Paula L. Dickey
(Type or print name of person mailing paper)

Paula L. Dickey
(Signature of person mailing paper)

PRELIMINARY AMENDMENT

Please enter the following amendment to the English Translation of the International Application before examining this application.

In the Specification

Please replace the Sequence Listing on pages 58-74 with the attached SUBSTITUTE SEQUENCE LISTING (pages 58-68). Please renumber the claims and Abstract pages accordingly.

In the Claims

Please cancel claims 2, 4, 6-9, 11, 12, 14-16, 19, 20, 22-25, 33, 36-38, 40, 42-51, and 53-57 without prejudice prior to calculating the filing fee for the instant application.

In claim 32, second line, after "according to" delete "one of claims 1 to 31" and insert therefor --claim 1--.

In claim 34, second line, before "characterized in" delete "or 33".

In claim 35, third line, after "as defined in" delete "claims 1 to 31" and insert therefor --claim 1--.

In claim 41, third line, after "according to" delete "claims 1 to 31" and insert therefor --claim 1--.

In claim 52, second line, after "according to" delete "one of claims 41-51" and insert therefor --claim 41--.

REMARKS

Claims 2, 4, 6-9, 11, 12, 14-16, 19, 20, 22-25, 33, 36-38, 40, 42-51, and 53-57 have been cancelled. No new matter has been added.

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Collegeville, PA 19426-0997
Telephone: (610) 454-3839
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Dated: Sept. 29, 1997

Respectfully submitted,

Paul F. Fehlner, Ph.D.
Attorney for Applicants
Registration No. 35,135

SUBSTTUTE
SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: BRACCO, Laurent
SCHWEIGHOFFER, Fabien
TOCQUE, Bruno

(ii) TITLE OF INVENTION: CONDITIONAL EXPRESSION SYSTEM

(iii) NUMBER OF SEQUENCES: 29

(iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: Rhone-Poulenc Rorer Inc.
(B) STREET: 500 Arcola Road, Mailstop 3C43
(C) CITY: Collegeville
(D) STATE: PA
(E) COUNTRY: USA
(F) ZIP: 19426

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE:
(C) CLASSIFICATION:

(vii) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: FR 95/03841
(B) FILING DATE: 31-MAR-1995

(vii) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: WO PCT/FR96/00477
(B) FILING DATE: 29-MAR-1996

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: Fehlner Esq., Paul F.
(B) REGISTRATION NUMBER: 35,135
(C) REFERENCE/DOCKET NUMBER: ST95021-US

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: (610) 454-3839
(B) TELEFAX: (610) 454-3808

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 19 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

TCTCTATCAC TGATAGGGA 19

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 17 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

TATCACCGCA AGGGATA 17

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 74 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

```
Lys Lys Pro Leu Asp Gly Glu Tyr Phe Thr Leu Gln Ile Arg Gly Arg
1               5                  10                  15

Glu Arg Phe Glu Met Phe Arg Glu Leu Asn Glu Ala Leu Glu Leu Lys
            20                  25                  30

Asp Ala Gln Ala Gly Lys Glu Pro Gly Gly Ser Arg Ala His Ser Ser
        35                  40                  45

His Leu Lys Ser Lys Lys Gly Gln Ser Thr Ser Arg His Lys Lys Leu
    50                  55                  60

Met Phe Lys Thr Glu Gly Pro Asp Ser Asp
65                  70
```

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 768 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

TTACTCGCGG CCCAGCCGGC CATGGCCCAG GTGCAGCTGC AGCAGTCTGG GGCAGAGCTT 60

GTAAGGTCAG GGGCCTCAGT CAAGTTGTCC TGCACAGCTT CTGGCTTCAA CATTAAAGAC 120

TACTATATGC ACTGGGTGAA GCAGAGGCCT GAACAGGGCC TGGAGTGGAT TGGATGGATT 180

GATCCTAAGA ATGGTGATAC TGAATATGCC CCGAAGTTCC AGGGCAAGGC CACTATGACT 240

GCAGACACAT CCTCCAATAC AGCCTACCTG CAGCTCAGCA GCCTGGCATC TGAGGACACT 300

GCCGTGTATT ATTGTAATTT TTACGGGGAT GCTTTGGACT ATTGGGGCCA AGGGACCACG 360

GTCACCGTCT CCTCAGGTGG AGGCGGTTCA GGCGGAGGTG GCTCTGGCGG TGGCGGATCG 420

GATGTTTTGA TGACCCAAAC TCCACTCACT TTGTCGGTTA CCATTGGACA ACCAGCCTCC 480

ATCTCTTGCA AGTCAAGTCA GAGCCTCTTG GATAGTGATG GAAAAACATA TTTGAATTGG 540

TTGTTACAGA GGCCAGGCCA GTCTCCAAAG CGCCTAATCT ATCTGGTGTC TAAACTGGAC 600

TCTGGAGTCC CTGACAGGTT CACTGGCAGT GGATCAGGGA CAGATTTCAC ACTTAAAATC 660

AACAGAGTGG AGGCTGAGGA TTTGGGAGTT TATTATTGCT GGCAAGGTAC ACATTCTCCG 720

CTTACGTTCG GTGCTGGCAC CAAGCTGGAA ATTAAACGGG CGGCCGCA 768

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

Gly Gly Gly Gly Ser Gly Gly Gly Gly Ser Gly Gly Gly Gly Ser
1 5 10 15

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 1..30

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

```
CCC AAG CCC AGT ACC CCC CCA GGT TCT TCA                                  30
Pro Lys Pro Ser Thr Pro Pro Gly Ser Ser
 1               5                  10
```

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 10 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

```
Pro Lys Pro Ser Thr Pro Pro Gly Ser Ser
 1               5                  10
```

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 1..18

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

```
ATG AAC CGG CTG GGC AAG                                                  18
Met Asn Arg Leu Gly Lys
                 15
```

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 6 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

Met Asn Arg Leu Gly Lys
1 5

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 33 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 1..33

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

GAA CAA AAA CTC ATC TCA GAA GAG GAT CTG AAT 33
Glu Gln Lys Leu Ile Ser Glu Glu Asp Leu Asn
10 15

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 11 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

Glu Gln Lys Leu Ile Ser Glu Glu Asp Leu Asn
1 5 10

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 7 amino acids
(B) TYPE: amino acid

(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

Pro Lys Lys Lys Arg Lys Val
1 5

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 76 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

GGCTCTAGAC CCAAGCCCAG TACCCCCCCA GGTTCTTCAA CGCGTGGATC CATGTCCAGA 60

TTAGATAAAA GTAAAG 76

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 51 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

CGTACGGAAT TCGGGCCCTT ACTCGAGGGA CCCACTTTCA CATTTAAGTT G 51

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 76 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

GGCTCTAGAC CCAAGCCCAG TACCCCCCCA GGTTCTTCAA CGCGTGGATC CATGGAACAA 60

CGCATAACCC TGAAAG 76

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 51 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

CGTACGGAAT TCGGGCCCTT ACTCGAGTGC TGTTGTTTTT TTGTTACTCG G 51

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 35 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

CAGGCCATGG CATGAAGAAA CCACTGGATG GAGAA 35

(2) INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 43 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

CGTCGGATCC TCTAGATGCG GCCGCGTCTG AGTCAGGCCC TTC 43

(2) INFORMATION FOR SEQ ID NO:19:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 31 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

CAGGCTCGAG AAGAAACCAC TGGATGGAGA A 31

(2) INFORMATION FOR SEQ ID NO:20:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 61 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

CAGGCTCGAG CCCAAGCCCA GTACCCCCCC AGGTTCTTCA AAGAAACCAC TGGATGGAGA 60

A 61

(2) INFORMATION FOR SEQ ID NO:21:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 37 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

GGTCGAATTC GGGCCCTCAG TCTGAGTCAG GCCCTTC 37

(2) INFORMATION FOR SEQ ID NO:22:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 29 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

CAGGCCATGG AGGAGCCGCA GTCAGATCC 29

(2) INFORMATION FOR SEQ ID NO:23:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 46 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

CGTCGGATCC TCTAGATGCG GCCGCCACGG GGGGAGCAGC CTCTGG 46

(2) INFORMATION FOR SEQ ID NO:24:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 66 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

```
Met Glu Gln Arg Ile Thr Leu Lys Asp Tyr Ala Met Arg Phe Gly Gln
1               5                  10                  15

Thr Lys Thr Ala Lys Asp Leu Gly Val Tyr Gln Ser Ala Ile Asn Lys
            20                  25                  30

Ala Ile His Ala Gly Arg Lys Ile Phe Leu Thr Ile Asn Ala Asp Gly
        35                  40                  45

Ser Val Tyr Ala Glu Glu Val Lys Pro Phe Pro Ser Asn Lys Lys Thr
    50                  55                  60
```

Thr Ala
65

(2) INFORMATION FOR SEQ ID NO:25:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 23 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:

GATCCTATCA CCGCAAGGGA TAA 23

(2) INFORMATION FOR SEQ ID NO:26:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 23 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:26:

GATAGTGGCG TTCCCTATTT CGA 23

(2) INFORMATION FOR SEQ ID NO:27:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 48 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:27:

GATCCGACTT TCACTTTTCT CTATCACTGA TAGTGAGTGG TAAACTCA 48

(2) INFORMATION FOR SEQ ID NO:28:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 48 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:28:

AGCTTGAGTT TACCACTCCC TATCAGTGAT AGAGAAAAGT GAAAGTCG 48

(2) INFORMATION FOR SEQ ID NO:29:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 96 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:29:

GATCCGACTT TCACTTTTCT CTATCACTGA TAGTGAGTGG TAAACTCACT AGGCTCAAAG 60

TGAAAAGAGA TAGTGACTAT CACTCACCAT TTGAGT 96

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CONDITIONAL EXPRESSION SYSTEM

U.S. National Stage of PCT/FR96/00477

English Translation of the International Application
(84 pages text; 10 sheets drawings)

Atty. Docket No. ST95021-US

CERTIFICATE OF MAILING (37 CFR § 1.10)

GB840711234 US
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CONDITIONAL EXPRESSION SYSTEM

The present invention relates to a new system for the conditional expression of genes. It also relates to the use of this system in gene or cell therapy, to target very selectively the expression of genes of interest.

Gene and cell therapies consist in correcting a deficiency or an abnormality (mutation, aberrant expression and the like) or in ensuring the expression of a therapeutic protein of interest by introducing a genetic information into the cell or affected organ. This genetic information can be introduced either ex vivo into a cell extracted from the organ, the modified cell then being reintroduced into the body (cell therapy), or directly in vivo in the appropriate tissue (gene therapy). Various techniques exist for carrying out the transfer of genes, among which are various tranfectional techniques involving natural or synthetic chemical or biochemical vectors such as complexes of DNA and DEAE-dextran (Pagano et al., J. Virol. 1 (1967) 891), of DNA and nuclear proteins (Kaneda et al., Science 243 (1989) 375), of DNA and lipids (Felgner et al., PNAS 84 (1987) 7413), the use of liposomes (Fraley et al., J. Biol. Chem. 255 (1980) 10431), cationic lipids and the like. Another technique is based on the use of viruses as vectors for the transfer of genes. In this regard, various viruses have been tested for their capacity to infect certain cell populations. In

particular, the retroviruses (RSV, HMS, MMS and the like), the HSV virus, the adeno-associated viruses and the adenoviruses. One of the major difficulties for the development of these gene and cell therapies lies, however, in the selectivity of the treatment. Depending on the applications, depending on the gene to be transferred, it is important to be able to target only certain tissues or certain parts of the body in order to concentrate the therapeutic effect and to limit dissemination and side effects. This targeting can be achieved by using vectors having a given cell specificity. Another approach consists in using expression signals which are specific for certain cell types. In this regard, the so-called specific promoters have been described in the literature, such as the promoter of the genes encoding pyruvate kinase, villin, GFAP, the promoter for the fatty acid-binding intestinal protein, the promoter for the α-actin of the cells of the smooth muscle, or the promoter of the apo-AI, apo-AII and human albumin genes, and the like. However, these promoters have certain disadvantages and in particular they have some transcriptional background noise which may be disruptive for the expression of toxic genes and they are limited to certain cells and cannot therefore be used for any application.

The present invention now describes a new conditional system for the expression of genes which is particularly selective and efficient. One of the

advantageous characteristics of the system of the invention lies in its capacity to express a gene not according to a type of cell, but according to the presence of a specific cellular element or a specific physiological situation. This system involves bispecific chimeric molecules comprising a domain capable of binding selectively a defined DNA sequence and a detecting domain capable of binding specifically a transactivator or a transactivating complex.

A first feature of the present invention consists more particularly in the creation and the expression of bispecific chimeric molecules comprising a domain capable of binding selectively a defined DNA sequence and a domain typical of binding specifically a transactivator or transrepressor or a transactivating or transrepressing complex.

Another feature of the present invention consists in a nucleic acid sequence encoding a chimeric molecule as defined above, as well as any expression vector comprising the said nucleic sequence.

Another feature of the invention consists in a conditional system for the expression of genes comprising (i) a chimeric molecule as defined above and (ii) an expression cassette comprising a regulatory sequence, a minimal promoter (whose activity depends on the presence of a transactivator) and the said gene.

Another feature of the invention also consists in an expression vector comprising

- a nucleic sequence encoding a chimeric molecule as defined above and

- the said expression cassette.

The conditional expression system of the invention is particularly appropriate for use in gene or cell therapy, to target very selectively the expression of genes of interest.

One of the components of the system of the invention therefore consists in specific bispecific chimeric molecules comprising a domain capable of binding selectively a defined DNA sequence and a domain capable of binding specifically a transactivator or a transactivating complex. The bispecificity of the molecules of the invention lies, on the one hand, in their capacity to bind a defined DNA sequence (generally designated operator or regulatory sequence) and, on the other hand, in their capacity to recruit specifically a transactivating or transrepressing protein domain which makes it possible to induce or to repress the expression of genes.

The invention relates more particularly to the development of bispecific chimeric molecules allowing the recruitment of any transcriptional factor whose activation or inactivation leads to a physiopathological situation. The bispecific chimeric molecules of the invention thus allow the selective recruitment of transcriptional transactivators which are specific for a physiopathological state, the

attachment of these transcriptional factors to promoters through the attachment of these molecules to defined DNA sequences located near these promoters (operator or regulatory sequences), and thus the conditional expression of genes (Figure 1).

The invention also relates to the development of bispecific chimeric molecules which allow the recruitment not of a molecule carrying a transactivating domain but of a transcriptional transactivating complex, that is to say a complex formed between a target molecule present in a cell and a molecule carrying a transactivating domain (Figure 2). In this case, the transactivating complex is preferably formed by means of a second bispecific chimeric molecule comprising a transactivating domain and a selective domain for binding to the said cellular molecule. The attachment of this second molecule allows the formation of a transcriptional transactivating binary complex, which complex then being recruited by the detecting system of the invention. The attachment of this ternary complex close to promoters thus allows the regulated expression of genes. This type of construct makes it possible advantageously to extend the conditions for using the system of the invention to the detection of any intracellular molecule lacking a transactivating domain, whether it is an endogenous molecule or a molecule of infectious origin for example.

The system of the invention thus makes it possible, by virtue of a very selective detection system ("sensor"), to activate the expression of genes of interest only in the presence of target proteins. These may be transcriptional factors which appear during physiological or physiopathological situations, or any endogenous molecule or any molecule of infectious origin for example. The system of the invention contains, indeed, a very sensitive and very selective detecting component which makes it possible to base the expression of a gene on the presence, the appearance or the disappearance of any molecule in a cell.

Within the framework of the present invention, the term transactivator designates any transcription transactivating factor or any protein comprising a transcriptional transactivating domain. The transactivating complex designates the complex formed between a molecule present in a cell and a bispecific molecule of the invention comprising a transactivating domain and a domain for specific binding to the said molecule. The expression system of the invention can be used to recruit any transactivating protein or any protein carrying a transactivating domain, and in particular any protein of viral, parasitic, mycobacterial or cellular origin possessing a transcriptional transactivating activity. Among the transcriptional factors of viral origin,

there may be mentioned especially the Tat protein of the HIV virus, the E6/E7 proteins of the papilloma virus or alternatively the EBNA protein of the Epstein-Barr virus. These proteins possess a transcriptional transactivating domain and are present only in the cells infected by these viruses, that is to say under specific physiopathological conditions. The conditional expression system according to the invention allows advantageously a detection of this physiological situation (the appearance of these transactivators which are specific for the viral infection) and the induction of a selective expression of (a) given gene(s). Among the cellular proteins, there may be mentioned preferably the mutated or wild-type p53 protein. The p53 protein consists of 393 amino acids. In its wild-type form, it is a tumour suppressor capable of negatively regulating cell division and growth. This activity is linked to the presence of a transcriptional transactivating domain in the structure of the p53 protein, located in the N-terminal region of the protein (residues 1-100 approximately). In certain situations, the wild-type p53 is also capable of inducing apoptosis (Yonish-Rouach et al., Nature, 352, 345-347, 1991). Given that these properties are manifested in a stress situation where the integrity of the cellular DNA is threatened, it has been suggested that p53 is a "guardian of the genome". The presence of mutated p53's in about 40% of human tumours, all types

included, reinforces this hypothesis and underlines the probably critical role which mutations of this gene play in the tumour development (for reviews see Montenarh, Oncogene, 7, 1673-1680, 1992; Oren, FASEB J., 6, 3169-3175, 1992; Zambetti and Levine, FASEB J., 7, 855-865, 1993). Within the framework of the present invention, it is possible to recruit selectively the transactivating domain of the p53 protein and thus to induce the controlled expression of (a) gene(s) only in cells containing this protein. It is particularly advantageous according to the invention to recruit specifically the mutated forms of the p53 protein which, as indicated above, appear in physiopathological situations of cell hyperproliferation (cancer type). This targeting may be achieved preferably by means of a binding domain specific for the mutated forms of the p53 protein. There is, however, a defacto specificity linked to the accumulation of the mutated forms which possess a half-life which is much greater than the wild-type form.

The system of the invention can also be used to induce the selective expression of (a) gene(s) by detecting any target molecule present in a cell. The detected protein is preferably a protein which appears in a cell in abnormal situations (infection, hyperproliferation and the like). They may be in particular viral proteins such as structural or functional proteins of a virus, and especially of the

HIV, hepatitis or herpes virus and the like. They may also be proteins which are specific for a cell hyperproliferation state, such as especially the myc, fos and jun proteins, the cyclines and the like.

One of the properties of the chimeric molecules of the invention therefore consists in their capacity to bind to specific DNA regions (operator or regulatory regions). This binding makes it possible to bring the transactivating domain close to the promoter and as a result to activate the expression of a gene placed under the control of the said promoter.

The domain capable of binding selectively a defined sequence of DNA present in the molecules of the invention is essentially of protein origin. More preferably, this domain is derived from a prokaryotic or eukaryotic protein capable of interacting with DNA sequences. Numerous genetic and structural studies have now made it possible to define precisely, within proteins interacting with double-stranded DNA sequences, the domains responsible for these interactions.

Among the prokaryotic proteins which interact with double-stranded DNA sequences, there may be mentioned especially bacterial repressors and, preferably, the E. coli tetracycline repressor and the Cro repressor of the lambda bacteriophage.

The E. coli tetracycline repressor (tetR) is a protein of about 210 amino acids. In E. coli, tetR

negatively controls the transcription of genes mediating the resistance to this antibiotic within the tet operon. In the absence of tetracycline, the tetR repressor attaches to the DNA at the level of a specific sequence (designated operator sequence or Tetop) and represses the transcription of the resistance gene. In contrast, in the presence of tetracycline, the tetR repressor no longer attaches to the tetop operator allowing a constitutive transcription of the gene (Hillen, W. and Wissman, A. (1989) in Protein-Nucleic Acid Interaction. Topics in Molecular and Structural Biology. eds, Saenger, W. and Heinemann, U. (Macmillan, London), Vol. 10, pp. 143-162). The tetR sequence has been published (it is reproduced especially in WO94/04682). The specific double-stranded DNA sequence for the binding of tetR to the DNA (Tetop) is composed of the following unit:

TCTCTATCACTGATAGGGA (SEQ ID No. 1).

This unit can be repeated several times in order to increase the affinity and the efficiency of the system. Thus, the regulatory sequence may comprise up to 10 units and, preferably, contains 2 units (Tetop2) or 7 units (Tetop7) (see Figure 3).

The Cro protein was initially defined as a regulator of the expression of the CI repressor (Eisen, H. et al (1970) PNAS 66, pp. 855). The cloning of the cro gene has allowed the identification of a protein of 66 amino acids (SEQ ID No. 21; Roberts, T.

et al (1977) Nature 270, pp. 274). Cro exerts its physiological role by attaching preferably to the lambda OR_3 operator.

The specific double-stranded DNA sequence for the binding of Cro to the DNA (region designated OR3) is composed of the following bases:

TATCACCGCAAGGGATA (SEQ ID No. 2)

This region can also be repeated several times in order to increase the affinity and the efficiency of the system (see Figure 4).

Among the eukaryotic proteins interacting with double-stranded DNA sequences, the proteins or domains derived from the STAT, p53 or NFkB proteins are preferably used for the construction of the molecules of the invention (Inoue et al., PNAS 89 (1992) 4333). As regards the p53 protein, its DNA-binding domain is located in the central region of the protein and, more precisely, the region between amino acids 102 and 292 (Pavletich et al., Genes & Dev. 7 (1993) 2556).

As indicated above, the domain capable of binding selectively a defined sequence of DNA present in the molecules of the invention is preferably derived from a prokaryotic or eukaryotic protein capable of interacting with a double-stranded DNA region. The domain used for the construction of the molecules of the invention may consist of the entire protein or of a fragment thereof comprising the region for interaction with the DNA. This domain has been identified for

various proteins and especially TetR (see for example Berens et al., J. Biol. Chem. 267 (1992) 1945). It may also consist of a derivative of this protein or of the fragment having conserved the DNA-binding properties. Such derivatives are especially proteins exhibiting modifications of one or more amino acids, for example in order to allow their fusion with the other domains of the molecules of the invention, which are prepared according to conventional molecular biology techniques. Derivatives of the TetR and Cro proteins, for example, have been described in the literature, which possess point mutations and/or deletions (Hecht et al., J. Bact. 175 (1993) p. 1206; Altschmied et al., EMBO J 7 (1988) 4011; Baumeister et al., Proteins 14 (1992) 168; Hansen et al., J. Biol. Chem. 262 (1987) 14030). The capacity of these derivatives for binding to a defined DNA sequence can then be tested by incubating the derivative prepared with the regulatory sequence and detecting the complexes formed. In addition, the derivatives may also be proteins having enhanced DNA-binding properties (specificity, affinity and the like).

According to a preferred embodiment, the domain capable of binding selectively a defined sequence of DNA present in the molecules of the invention is derived from a prokaryotic protein. This type of construct is particularly advantageous since these proteins, of nonhuman origin, recognize double-

stranded DNA sites of at least 14 nucleotides. The probability of finding the same sequence within the human genome is practically zero and therefore the expression system obtained is all the more selective.

In a preferred embodiment, the domain capable of binding selectively a defined sequence of DNA present in the molecules of the invention is derived from the tetR or Cro proteins. It is most particularly advantageous to use the complete tetR or Cro proteins (SEQ ID No. 21).

The domain capable of binding specifically the transcriptional transactivator or the transcriptional transactivating complex present in the molecules of the invention may be of various types. It may be in particular an oligomerizing domain in the case where the transactivator or the transactivating complex targeted also comprises such a domain. It may also be any synthetic or natural domain known to interact with the said transactivator or transactivating complex. It may alteratively be an antibody or a fragment or derivative of an antibody directed against the transactivator or transactivating complex.

Among the oligomerizing domains which can be used within the framework of the invention, there may be mentioned more particularly the leucine zippers, the SH2 domains and the SH3 domains for example. The leucine zippers are amphipatic α helices which contain

4 or 5 leucines every 7 amino acids. This periodicity allows the localization of the leucines roughly at the same position on the α helix. The dimerization is sustained by hydrophobic interactions between the side chains of the leucines of two contiguous zipper domains (Vogt et al., Trends in Bioch. Science 14 (1989) 172). The SH2 domains are known to interact with specific peptide sequences phosphorylated on tyrosine. The SH3 domains can be used to form an oligomer with any transactivator or transactivating complex comprising the corresponding proline-rich peptide (Pawson et al., Current Biology 3 (1993) 434). It is also possible to use protein regions known to induce oligomerization, such as especially the C-terminal region of the p53 protein. The use of this region makes it possible to recruit selectively the p53 proteins present in a cell. A p53 region between amino acids 320-393 (SEQ ID No. 3), 302-360 or 302-290 is preferably used within the framework of the invention.

Among the synthetic or natural domains known to interact with the molecule comprising the targeted transactivating component, there may be mentioned for example the region of the MDM2 protein which interacts with the p53 protein. This type of construct thus makes it possible to recruit, as transactivator, the wild-type or mutated p53 protein.

A preferred domain for the specific binding to the transcriptional transactivator of the invention

consists of an antibody or an antibody fragment or derivative. The antibody fragments or derivatives are for example the Fab or F(ab)'2 fragments, the VH or VL regions of an antibody or alternatively single-chain antibodies (ScFv) comprising a VH region linked to a VL region by an arm. This type of domain is particularly advantageous since it can be directed against any molecule.

Antibodies, molecules of the immunoglobulin superfamily, consist of different chains (2 heavy chains (H) and 2 light chains (L)) which are themselves composed of different domains (variable domain (V), joining domain (J), and the like). The domain for binding to the transactivator or the transactivating complex which is present in the molecules of the invention advantageously consists of an antibody fragment or derivative comprising at least the antigen-binding site. This fragment may be either the variable domain of a light chain (V_L) or of a heavy chain (V_H), optionally in the form of an Fab or F(ab)'2 fragment or, preferably, in the form of single-chain antibodies (ScFv). The single-chain antibodies used for the construction of the molecules of the invention consist of a peptide corresponding to the binding site of the variable region of the light chain of an antibody linked by a peptide arm to a peptide corresponding to the binding site of the variable region of the heavy chain of an antibody. The construction of nucleic acid

sequences encoding such modified antibodies according to the invention has been described for example in patent US 4,946,778 or in applications WO94/02610, WO94/29446. It is illustrated in the examples.

A preferred construct according to the present invention comprises a domain for binding to a p53 protein. It is more preferably an antibody derivative directed against a p53 protein. A specific embodiment consists of a single-chain antibody directed against p53. By way of specific example, the ScFv of sequence SEQ ID No. 4 is used whose construction is described in the examples.

The DNA binding domain and the transactivator-binding domain are generally linked to each other through an arm. This arm generally consists of a peptide which confers sufficient flexibility for the two domains of the molecules of the invention to be functional autonomously. This peptide is generally composed of uncharged amino acids, which do not interfere with the activity of the molecules of the invention, such as for example glycine, serine, tryptophan, lysine or proline. The arm generally comprises from 5 to 30 amino acids and, preferably, from 5 to 20 amino acids. Examples of peptide arms which can be used for the construction of the molecules of the invention are for example:

- GGGGSGGGGSGGGGS (SEQ ID No. 5)
- PKPSTPPGSS (SEQ ID No. 6) whose coding

sequence is CCCAAGCCCAGTACCCCCCCAGGTTCTTCA (SEQ ID No. 6).

Preferred examples of molecules according to the invention are especially the following molecules:

a) ScFv-tag-Hinge-TET or Cro (Figure 5A)

This type of molecule comprises:

- a domain for binding to a transactivator consisting of a single-chain antibody,

- a tag peptide sequence recognized by a monoclonal antibody allowing the immunological detection of the molecule. This sequence may be for example the VSV epitope of the MNRLGK sequence (SEQ ID No. 7) whose coding sequence is ATGAACCGGCTGGGCAAG (SEQ ID No. 7) or the moic epitope of the sequence EQKLISEEDLN (SEQ ID No. 8) whose coding sequence is GAACAAAAACTCATCTCAGAAGAGGATCTGAAT (SEQ ID NO. 8), which is recognized by the antibody 9E10.

- a peptide arm of sequence SEQ ID No. 6 (Hinge) and

- a DNA-binding domain consisting of the TET or Cro protein. Preferably, ScFv is directed against a p53 protein.

b) ScFv-Hinge-TET or Cro (Figure 5B)

This type of molecule comprises the same elements as the molecule a) except for the tag sequence which is absent.

c) ScFV-TET or Cro (Figure 5C)

This type of molecule comprises simply a

domain for binding to a transactivator consisting of a single-chain antibody and a DNA-binding domain consisting of the TET or Cro protein. It contains neither an arm nor a tag sequence. In this construct, the transactivator-binding domain is located in the N-terminal part of the molecule and the DNA-binding domain in the C-terminal part.

d) TET or Cro-ScFv (Figure 5D)

This type of molecule is similar to the c) type above. The difference is essentially in the arrangement of the domains: the transactivator-binding domain is now located in the C-terminal part of the molecule and the DNA-binding domain in the N-terminal part.

e) TET or Cro-Hinge-ScFv (Figure 5E)

This type of molecule comprises the same elements as the b) molecule above. The difference is essentially in the arrangement of the domains: the transactivator-binding domain is now located in the C-terminal part of the molecule and the DNA-binding domain in the N-terminal part.

f) Oligom-tag-Hinge-TET or Cro (Figure 5A)

This type of molecule is similar to the a) type, with the exception of the transactivator-binding domain which is replaced by the domain for oligomerization to the p53 protein of sequence SEQ ID No. 3. This molecule makes it possible to recruit the mutated p53 proteins which appear in tumour cells.

g) Oligom-Hinge-TET or Cro (Figure 5B)

This type of molecule is similar to the b) type, with the exception of the transactivator-binding domain which is replaced by the domain for oligomerization to the p53 protein of sequence SEQ ID No. 3.

h) Oligom-TET or Cro (Figure 5C)

This type of molecule is similar to the c) type, with the exception of the transactivator-binding domain which is replaced by the domain for oligomerization to the p53 protein of sequence SEQ ID No. 3.

i) TET or Cro-Oligom (Figure 5D)

This type of molecule is similar to the d) type, with the exception of the transactivator-binding domain which is replaced by the domain for oligomerization to the p53 protein of sequence SEQ ID No. 3.

j) TET or Cro-Hinge-Oligom (Figure 5E)

This type of molecule is similar to the e) type, with the exception of the transactivator-binding domain which is replaced by the domain for oligomerization to the p53 protein of sequence SEQ ID No. 3.

Moreover, in each of these molecules, the peptide arm can be easily replaced by the sequence (G4S)3 (SEQ ID No. 5).

Another subject of the present invention

consists in a nucleic acid sequence encoding a chimeric molecule as defined above. It is advantageously a DNA, especially a cDNA, sequence. It may also be an RNA. The sequences of the invention are generally constructed by assembling, within a cloning vector, the sequences encoding the various domains according to conventional molecular biology techniques. The nucleic acid sequences of the invention can optionally be modified chemically, enzymatically or genetically, in order to generate domains which are stabilized, and/or multifunctional, and/or of reduced size, and/or with the aim of promoting their location in such or such intracellular compartment. Thus, the nucleic acid sequences of the invention may comprise sequences encoding nuclear localisation peptides (NLS). In particular, it is possible to fuse the sequences of the invention with the sequence encoding the SV40 virus NLS, whose peptide sequence is the following: PKKKRKV (SEQ ID No. 9) (Kalderon et al., Cell 39 (1984) 499).

The nucleic sequences according to the invention are advantageously part of an expression vector, which may be of plasmid or viral nature.

Another subject of the present invention consists in a fusion protein comprising a transcriptional transactivator domain and a domain for specific binding to a given molecule, optionally linked by a peptide arm, as well any nucleic acid sequence encoding such a fusion. The transactivator domain may

be derived from any transcriptional transactivator protein, such as p53, VP16, EBNA, Et/E7, Tat, and the like.

Another subject of the invention consists of a conditional system for the expression of genes comprising:

- a chimeric molecule as defined above and
- an expression cassette comprising a regulatory sequence, a minimal transcriptional promoter and the said gene.

The expression cassette contains the components necessary for the activation of the expression of the gene by the transactivator or transactivating complex recruited by the bispecific molecule. Thus, the regulatory sequence is the DNA binding sequence of the chimeric molecule expressed. When the DNA-binding domain of the chimeric molecule is represented by all or part of TetR, the regulatory sequence comprises the sequence SEQ ID No. 1 or a derivative thereof, optionally repeated several times. It is preferably the op2 (comprising 2 repeated Tetop units) or Op7 sequence (comprising 7 repeated Tetop units, as described for example in Weinmann et al., The Plant Journal 5 (1994) 559). Likewise, when the DNA-binding domain of the chimeric molecule is represented by all or part of Cro, the regulatory sequence comprises the sequence SEQ ID No. 2 or a derivative thereof, optionally repeated several times. It is

preferably the sequence OR3. The derivatives of the sequences SEQ ID No. 1 and 2 may be any sequence obtained by a modification of genetic nature (mutation, deletion, addition, repetition and the like) and conserving the capacity to bind specifically a protein. Such derivatives have been described in the literature (Baumeister et al., cited above, Tovar et al., Mol. Gen. Genet. 215 (1988) 76, WO94/04672).

As regards the minimal transcriptional promoter, it is a promoter whose activity depends on the presence of a transactivator. Because of this, in the absence of the chimeric molecule, the promoter is inactive and the gene is not or is barely expressed. On the other hand, in the presence of a chimeric molecule, the transactivator or transactivating complex recruited makes it possible to induce the activity of the minimal promoter and thus the expression of the gene of interest. The minimal promoter generally consists of an INR or a TATA box. These components are indeed the minimum components necessary for the expression of a gene in the presence of a transactivator. The minimal promoter can be prepared from any promoter by genetic modification. By way of preferred example of a candidate promoter, there may be mentioned the promoter of the thymidine kinase gene. Advantageous results have more precisely been obtained with a minimal promoter derived from the TK promoter composed of nucleotides -37 to +19. The minimal promoter can also be derived

from human CMV. In particular, it may consist of the fragment between nucleotides -53 and +75 or -31 and +75 from CMV. Any conventional promoter can however be used such as, for example the promoter of the genes encoding chloramphenicol acetyltransferase, β-galactosidase or alternatively luciferase.

The expression cassette advantageously consists of the following elements:

- as regulator sequence, a sequence comprising the sequence SEQ ID No. 1 or 2 or a derivative thereof, optionally repeated several times,
- as minimal promoter, a promoter derived from the promoter of the thymidine kinase (TK) gene,
- a coding sequence of interest.

Still more preferably, the minimal promoter consists of the -37 to +19 region of the promoter of the thymidine kinase gene.

Advantageously, the expression cassette is chosen from the cassettes of structure Tetop2.TK-Gene; Tetop7.TK-Gene or OR3.TK-Gene.

Another feature of the invention consists in an expression vector comprising a nucleic acid sequence encoding a chimeric molecule and an expression cassette as defined above. In the vectors of the invention, the nucleic acid sequence encoding the chimeric molecule and the expression cassette may be inserted in the same orientation or in opposite orientations. Moreover, the vector may be of plasmid or viral nature.

Among the viral vectors, there may be mentioned more particularly the adenoviruses, the retroviruses, the herpes viruses or alternatively the adeno-associated viruses. The viruses according to the invention are defective, that is to say are incapable of replicating autonomously in the target cell. Generally, the genome of the defective viruses used within the framework of the present invention therefore lacks at least sequences necessary for the replication of the said virus in the infected cell. These regions may be either removed (completely or partly) or made nonfunctional, or substituted by other sequences and especially by the sequences of the invention. Preferably, the defective virus conserves, nevertheless, the sequences of its genome which are necessary for the encapsidation of the viral particles.

As regards more particularly adenoviruses, various serotypes, whose structure and properties vary somewhat, have been characterized. Among these serotypes, the use of the type 2 or 5 human adenoviruses (Ad 2 or Ad 5) or the adenoviruses of animal origin (see application WO94/26914) is preferred within the framework of the present invention. Among the adenoviruses of animal origin which can be used within the framework of the present invention, there may be mentioned the adenoviruses of canine, bovine, murine (example: MAV1, Beard et al., Virology 75 (1990) 81), ovine, porcine, avian or alternatively simian

(example: SAV) origin. Preferably, the adenovirus of animal origin is a canine adenovirus, more preferably a CAV2 adenovirus [manhattan strain or A26/61 (ATCC VR-800) for example]. Preferably, adenoviruses of human or canine or mixed origin are used within the framework of the invention.

Preferably, the genome of the recombinant adenoviruses of the invention comprises at least the ITRs and the encapsidation region of an adenovirus, and the nucleic acid sequences encoding a chimeric molecule and an expression cassette as defined above. More preferably, in the genome of the adenoviruses of the invention, the E1 region at least is nonfunctional. The viral gene considered may be rendered nonfunctional by any technique known to a person skilled in the art, and especially by total suppression, by substitution (for example by the sequences of the invention), partial deletion, or addition of one or more bases in the gene(s) considered. Such modifications can be obtained in vitro (on the isolated DNA) or in situ, for example, by means of genetic engineering techniques, or alternatively by treating with mutagenic agents. Other regions can also be modified, and especially the E3 (WO95/02697), E2 (WO94/28938), E4 (WO94/28152, WO94/12649, WO95/02697) and L5 WO95/02697) region. According to a preferred embodiment, the adenovirus according to the invention comprises a deletion in the E1 and E4 regions. According to another preferred

embodiment, it comprises a deletion in the E1 region at the level of which are inserted the E4 region and the sequences of the invention (cf. FR 94 13355).

The defective recombinant adenoviruses according to the invention can be prepared by any technique known to a person skilled in the art (Levrero et al., Gene 101 (1991) 195, EP 185 573; Graham, EMBO. J. 3 (1984) 2917). In particular, they can be prepared by homologous recombination between an adenovirus and a plasmid carrying, inter alia, the DNA sequences of the invention (sequence encoding the chimeric molecule + expression cassette). The homologous recombination occurs after co-transfection of the said adenoviruses and plasmid into an appropriate cell line. The cell line used should preferably (i) be transformable by the said elements, and (ii) contain the sequences capable of complementing the defect adenovirus genome part, preferably in integrated form in order to avoid the risks of recombination. By way of example of a line, there may be mentioned the human embryonic kidney line 293 (Graham et al., J. Gen. Virol. 36 (1977) 59) which contains especially, integrated into its genome, the left-hand part of the genome of an Ad5 adenovirus (12%) or lines capable of complementing the E1 and E4 functions as described especially in applications Nos. WO 94/26914 or WO95/02697.

Next, the adenoviruses which have multiplied

are recovered and purified according to conventional molecular biology techniques, as illustrated in the examples.

As regards the adeno-associated viruses (AAV), they are relatively small DNA viruses which integrate in the genome of the cells which they infect, in a stable and site-specific manner. They are capable of infecting a broad spectrum of cells, without inducing any effect on cell growth, morphology or differentiation. Moreover, they do not seem to be involved in pathologies in man. The genome of the AAVs has been cloned, sequenced and characterized. It comprises about 4700 bases, and contains, at each end, an inverted repeat region (ITR) of about 145 bases, which serves as replication origin for the virus. The remainder of the genome is divided into 2 essential regions carrying the encapsidation functions: the left-hand part of the genome, which contains the rep gene involved in the viral replication and the expression of the viral genes; the right-hand part of the genome, which contains the cap gene encoding the virus capsid proteins.

The use of AAV- derived vectors for the transfer of genes in vitro and in vivo has been described in the literature (see especially WO91/18088, WO93/09329, US 4,797,368, US 5,139,941, EP 488 528). These applications describe various AAV-derived constructs in which the rep and/or cap genes are

deleted and replaced by a gene of interest, and their use for the transfer in vitro (on cells in culture) or in vivo (directly in an organism) of the said gene of interest. The defective recombinant AAVs according to the invention can be prepared by co-transfection, into a cell line infected by a human helper virus (for example an adenovirus), of a plasmid containing the nucleic sequences of the invention (sequence encoding the chimeric molecule + expression cassette) bordered by two AAV inverted repeat regions (ITR), and of a plasmid carrying the AAV encapsidation genes (rep and cap genes). The recombinant AAVs produced are then purified by conventional techniques.

As regards the herpes viruses and the retroviruses, the construction of recombinant vectors has been widely described in the literature: see especially Breakfield et al., New Biologist 3 (1991) 203; EP 453242, EP 178220, Bernstein et al., Genet. Eng. 7 (1985) 235; McCormick, BioTechnology 3, (1985) 689, and the like.

In particular, the retroviruses are integrative viruses which selectively infect dividing cells. They therefore constitute vectors of interest for cancer applications. The genome of the retroviruses essentially comprises two LTRs, an encapsidation sequence and three coding regions (gag, pol and env). In the recombinant vectors derived from retroviruses, the gag, pol and env genes are generally deleted,

completely or partly, and replaced by a heterologous nucleic acid sequence of interest. These vectors can be prepared from various types of retrovirus such as especially MoMuLV ("murine moloney leukaemia virus"; also called MoMLV), MSV ("murine moloney sarcoma virus"), HaSV ("harvey sarcoma virus"); SNV ("spleen necrosis virus"); RSV ("Rous sarcoma virus") or alternatively Friend's virus.

To construct recombinant retroviruses according to the invention, a plasmid comprising especially the LTRs, the encapsidation sequence and the sequences of the invention (sequence encoding the chimeric molecule + expression cassette) is generally constructed, then used to transfect a so-called encapsidation cell line capable of providing in trans the retroviral functions which are deficient in the plasmid. Generally, the encapsidation lines are therefore capable of expressing the gag, pol and env genes. Such encapsidation lines have been described in the prior art, and especially the PA317 line (US 4,861,719); the PsiCRIP line (WO90/02806) and the GP+envAm-12 line (WO89/07150). Moreover, the recombinant adenoviruses may contain modifications in the LTRs so as to suppress the transcriptional activity, as well as extended encapsidation sequences, comprising part of the gag gene (Bender et al., J. Virol. 61 (1987) 1639). The recombinant retroviruses produced are then purified by conventional techniques.

An example of the construction of a defective recombinant virus according to the invention (retrovirus) is described in Figure 8. This figure underlines a second advantage of the constructs according to the invention which consists in the absence of expression of the gene of interest in the encapsidation lines. Given that these lines lack the transactivator or the transactivating complex recruited by the system of the invention, the promoter is inactive and the gene is not expressed in the production cell (Figure 8A). It is only when the virus has effectively infected a target cell, that is to say a cell in which the transactivator or transactivating complex recruited by the system of the invention is present, that the gene is effectively expressed (Figure 8B). This is particularly advantageous for the construction of viruses containing genes whose expression would be toxic for the cells (Grb3-3, IL-2, and diphtheria toxine genes and the like).

For the implementation of the present invention, it is most particularly advantageous to use a defective recombinant retrovirus or adenovirus. These vectors possess, indeed, properties which are particularly advantageous for the transfer of genes into tumour cells.

Various types of non-viral vectors can also be used within the framework of the invention. The conditional expression system according to the

invention can indeed be incorporated into a non-viral agent capable of promoting the transfer and the expression of nucleic acids in eukaryotic cells. The chemical or biochemical vectors represent an advantageous alternative to the natural viruses, in particular for reasons of convenience, safety and also by the absence of a theoretical limit as regards the size of the DNA to be transfected.

These synthetic vectors have two principal functions, to compact the nucleic acid to be transfected and to promote its cellular attachment as well as its passage through the plasma membrane and, where appropriate, the two nuclear membranes. In order to overcome the polyanionic nature of the nucleic acids, the non-viral vectors all possess polycationic charges.

Among the synthetic vectors developed, the cationic polymers of the polylysine, (LKLK)n, (LKKL)n, poly(ethylenimine) and DEAE dextran type or alternatively the cationic lipids or lipofectants are the most advantageous. They possess the properties of condensing the ADN and of promoting its association with the cell membrane. Among the latter, there may be mentioned the lipopolyamines (lipofectamine, transfectam and the like) and various cationic or neutral lipids (DOTMA, DOGS, DOPE and the like). More recently, the concept of targeted transfection, mediated by a receptor, has been developed, which takes

advantage of the principle of condensing DNA by virtue of the cationic polymer while directing the attachment of the complex to the membrane by virtue of a chemical coupling between the cationic polymer and the ligand of a membrane receptor, which is present at the surface of the cell type which it is desired to graft. The targeting of the transferrin or insulin receptor or of the receptor for the asialoglycoproteins of the hepatocytes has thus been described.

The subject of the present invention is also any pharmaceutical composition comprising a vector as defined above. These compositions may be formulated for topical, oral, parental, intranasal, intravenous, intramuscular, subcutaneous, or intraocular administration and the like. Preferably, the composition according to the invention contains vehicles which are pharmaceutically acceptable for an injectable formulation. These may be isotonic sterile saline solutions (monosodium or disodium phosphate, sodium potassium, calcium or magnesium chloride and the like, or mixtures of such salts), or dry, especially freeze-dried, compositions which, upon addition, depending on the case, of sterilized water or of physiological saline, allow the preparation of injectable solutions. As regards the retroviruses, it may be advantageous to use directly the encapsidation cells or cells infected ex vivo and designed to be reimplanted in vivo, optionally in the form of neo-

organs (WO94/24298).

The doses of vector which are used for the injection may be adjusted according to various parameters, and especially according to the mode of administration used, the relevant pathology or alternatively the desired duration of treatment. In general the recombinant viruses according to the invention are formulated and administered in the form of doses of between 10^4 and 10^{14} pfu/ml. For the AAVs and the adenoviruses, doses of 10^6 and 10^{10} pfu/ml can also be used. The term pfu ("plaque forming unit") corresponds to the infectivity of a suspension of virions and is determined by infecting an appropriate cell culture, and measuring, generally after 48 hours, the number of plaques of infected cells. The techniques for examining the pfu titre of a viral solution are well documented in the literature.

The expression system according to the invention and the corresponding vectors are particularly useful for controlling the expression of genes of interest in the context of cell or gene therapies. They can thus be used to control the expression of any coding sequence of interest, and especially a sequence encoding a therapeutic product whether it is a peptide, polypeptide, protein, ribonucleic acid and the like. More particularly, the gene is a DNA sequence (cDNA, gDNA, synthetic DNA, of human, animal or plant origin, and the like) which

encodes a protein product such as enzymes, blood derivatives, hormones, lymphokines: interleukins, interferons, TNF, and the like (FR 9,203,120) growth factors, neurotransmitters or precursors thereof or synthesis enzymes, trophic factors: BDNF, CNTF, NF, IGF, GMF, aFGF, bFGF, NT3, NT5, and the like; apolipopropteins: ApoAl, ApoAIV, ApoE, and the like (FR 93 05125), dystrophin or a minidystrophin (FR 9,111,947), tumour suppressor genes: p53, Rb, Rap1A, DCC, k-rev, and the like (FR 93 04745), genes encoding factors involved in coagulation: Factors VII, VIII, IX, and the like, or alternatively all or part of a natural or artificial immunoglobulin (Fab, ScFv, and the like), an RNA ligand (WO91/19813) and the like.

The gene of interest may also be an antisense sequence whose expression in the target cell makes it possible to control the expression of genes or the transcription of cellular mRNAs. Such sequences can for example be transcribed, in the target cell, into RNAs complementary to cellular mRNAs and thus block their translation into protein, according to the technique described in Patent EP 140 308.

The present invention is particularly adapted to the expression of sequences encoding toxic factors. They may be in particular poisons for the cells (diphtheria toxin, pseudomonas toxin, ricin A and the like), a product which induces sensitivity to an external agent (suicide genes: thymidine kinase,

cytosine deaminase and the like) or alternatively killer genes capable of inducing cell death (Grb3-3 (PCT/FR94/00542), ScFv anti-ras (WO04/29446) and the like). The system of the invention makes it possible, indeed, to produce vectors, especially viral vectors, containing these sequences without toxicity for the production cells, and then next to induce the expression of these toxic molecules selectively in target cells having the desired transactivator or transactivating complex. This type of construct is therefore particularly suitable for strategies or antitumour therapies for example, in which the objective is to destroy selectively the affected cells. This system is also particularly advantageous for the expression of cytokines, interferons, TNF or TGF for example, of which an uncontrolled production may have highly marked side effects.

The present invention will be described in greater detail with the aid of the following examples which should be considered as illustrative and non-limiting.

Legends to the figures

Figure 1: Representation of the conditional expression system according to the invention which allows the selective recruitment of a transactivator by means of an oligomerizing domain (A) or an ScFv (B).

Figure 2: Representation of the conditional expression

system according to the invention which allows the selective recruitment of a transactivating complex.

Figure 3: Representation of an expression cassette according to the invention comprising a regulatory sequence Tetop7, a minimal promoter (TATA box) and a gene (CAT).

Figure 4: Representation of an expression cassette according to the invention comprising a regulatory sequence OR3, a minimal promoter (TATA box) and a gene (CAT).

Figure 5: Representation of bispecific chimeric molecules according to the invention.

Figure 6: Construction of DNA sequences encoding bispecific chimeric molecules according to the invention.

Figure 7: Representation of control chimeric constructs.

Figure 8: Structure and function of a viral vector (retrovirus) according to the invention.

Figure 9: Study of the interaction between the hydbrid molecules of the invention and a regulatory sequence.

Figure 10: Study of the interaction between the hybrid molecules of the invention and various forms of the p53 protein.

Figure 11: Demonstration of the activation of the Tet-Luc cassette in SAOS-2 cells.

Figure 12: Demonstration of the activation of the Tet-Luc cassette in H358 cells.

General molecular biology techniques

The general molecular biology methods such as centrifugation of plasmid DNA in a caesium chloride-ethidium bromide gradient, digestions with restriction enzymes, gel electrophoresis, transformation in E. Coli, precipitation of nucleic acids and the like, are described in the literature (Maniatis et al., 1989).

The enzymes were provided by New-England Biolabs (Beverly, MA). For the ligations, the DNA fragments are separated according to their size on 0.8 to 1.5% agarose gels, purified by GeneClean (BIO101, LaJolla CA) and incubated overnight at 14°C in a 50 mM tris HCl buffer pH 7.4, containing 10 mM $MgCl_2$ 10 mM DTT, 2mM ATP, in the presence of T4 phage DNA ligase.

The PCR (Polymerase Chain Reaction) amplification was also performed according to Maniatis et al., 1989, with the following specifications:

- $MgCl_2$ concentration adjusted to 8 mM.
- Denaturing temperature 95°C, hybridization temperature 55°C, extension temperature 72°C. This cycle was repeated 25 times in a PE9600 Thermal cycler (Perkin Elmer, Norwalk CO).

The oligonucleotides are synthesized using the chemistry of phosphoramidites protected at the β position by a cyanoethyl group (Sinha et al., 1984, Giles 1985), with an applied biosystem model 394 automatic DNA synthesizer (Applied Biosystem, Foster

City CA), according to the manufacturer's recommendations.

The sequencing was carried out on double-stranded templates by the chain termination method using fluorescent primers. We used the sequencing Taq Dye Primer Kit from Applied Biosystem (Applied Biosystem, Foster City CA), according to the manufacturer's specifications.

Examples

Example 1: Construction of expression cassettes comprising a regulatory sequence, a minimal transcriptional promoter and a gene.

1.1. Construction of the plasmid pTETop7/CAT

The plasmid pTETop7/CAT contains the following components (Figure 3):

- a regulatory sequence consisting of a sequence for interaction with the tetracycline repressor TetR composed of 7 repeated Tetop motifs (SEQ ID No. 1);

- a minimal promoter derived from the promoter of the thymidine kinase gene (-37 to +19 region carrying the TATA box);

- the sequence encoding chloramphenicol acetyltransferase (CAT) under the control of the said minimal promoter.

This plasmid was constructed by cloning the SmaI-BglII fragment of the plasmid pUHD10-7 (WO 94/29442) into the plasmid pKK232-8 (Pharmacia) previously digested with SmaI and BamHI.

1.2. Construction of the plasmid pOR_3/CAT

The plasmid pOR3/CAT contains the following components (Figure 4):

- a regulatory sequence consisting of a sequence OR3 for interaction with the Cro repressor (SEQ ID No. 2);

- a minimal promoter derived from the

promoter of the thymidine kinase gene (-37 to +19 region carrying the TATA box);

- the sequence encoding chloramphenicol acetyltransferase (CAT) under the control of the said minimal promoter.

This plasmid was constructed in the following manner: the sequence OR3 for interaction with the Cro repressor was synthesized artificially. For that, the following two oligonucleotides were synthesized:

Oligo 5533 (SEQ ID No. 22): 5'-GATCCTATCACCGCAAGGGATAA-3'

Oligo 5534 (SEQ ID No. 23): 3'-GATAGTGGCGTTCCCTATTTCGA-5'

These two oligonucleotides were then hybridized in order to reconstitute the double-stranded sequence OR3 bordered by sequences allowing its orientated cloning as follows:

GATCCTATCACCGCAAGGGATAA

GATAGTGGCGTTCCCTATTTCGA

1.3 Construction of cassettes for the expression of toxic genes

The cassettes for the expression of toxic genes are obtained from the plasmids described above (1.1 and 1.2) by replacing the CAT sequence by the sequence encoding the toxic product, preferably the Grb3-3 gene (PCT/FR94/00542), the thymidine kinase gene, the gene encoding the diphtheria or pseudomonas toxin and the like.

Example 2: Construction of a single-chain antibody specific for p53

This single-chain antibody was constructed according to the following procedure:

- The cDNAs encoding the VH and VL regions were obtained from the hybridoma pAb421 which produces an anti-p53 antibody. For that, the total RNAs from the hybridoma were extracted and subjected to a reverse transcription reaction using random hexamers as primers. The use of this type of primer makes it possible to avoid the use of primers specific for immunoglobulins. The cDNA clones obtained have a sufficient length to clone the V regions. However, since they represent a small fraction of the total cDNAs present, a preliminary amplification reaction should be carried out in order to produce sufficient DNA for the cloning. For that, the cDNAs encoding the VH and VL regions were amplified separately. The primers used are oligonucleotides which hybridize at the level of the opposite ends of the variable regions of each chain (H and L). The product of amplification using the primers specific for the heavy chains is a fragment of about 340 base pairs. The product of amplification using the primers specific for the light chains is a fragment of about 325 base pairs.

- After purification the cDNAs encoding the VH and VL regions of the antibody were assembled into a single chain by means of a nucleotide arm (L). The

nucleotide arm was constructed such that one of the ends binds to the 3′ end of the cDNA encoding the VH region and the other to the 5′ end of the cDNA encoding the VL region. The sequence of the arm encodes the peptide SEQ ID No. 5. The assembled sequence of about 700 bp contains, in the form of an NcoI-NotI fragment, the VH-L-VL chain whose sequence is represented SEQ ID No. 4 (amino acids 9 to 241). This sequence also includes at the C-terminals the tag sequence of myc (residues 256 to 266).

Example 3: Construction of nucleic acid sequences encoding bispecific chimeric molecules containing a domain for binding to a transactivator consisting of a single-chain antibody (ScFv).

3.1. Construction of a plasmid comprising a sequence ScFv-myc-Hinge-TetR or Cro (Figures 5A and 6)

The NcoI-NotI fragment containing the cDNA encoding the anti-p53 ScFv was first cloned into a plasmid of the pUC19 type. The sequence encoding the VSV epitope (SEQ ID No. 7) or the myc epitope (SEQ ID No. 9) is inserted downstream of the fragment (Figure 6).

The sequences encoding the TetR and Cro proteins were then obtained as follows:

- The sequence encoding TetR was obtained by amplification from a template plasmid carrying the TetR sequence by means of the following oligonucleotides:

Oligo 5474 (SEQ ID No.10):

encoding ScFv and tag.

4.4. The plasmids p53 320/393-TetR or Cro (Figure 5C) were obtained by cloning the fragment amplified in 4.1. in the form of an NcoI-BamHI fragment into the corresponding sites of the plasmids described in Example 3.1., as substitution for the region encoding ScFv, tag and Hinge.

4.5. The plasmids tetR or Cro-p53 320/393 (Figure 5D) or tetR or Cro-Hinge-p53 320/393 (Figure 5E) were obtained by cloning fragments amplified by PCR on a plasmid carrying the cDNA for the wild-type human p53 with the aid of the oligos 5537/5539 or 5538/5539 digested with XhoI/EcoRI into the plasmids described in 3.1., previously digested with XhoI/EcoRI.

Oligo 5537 (SEQ ID No. 16):

CAGGCTCGAGAAGAAACCACTGGATGGAGAA

Oligo 5538 (SEQ ID No. 17):

CAGGCTCGAGCCCAAGCCCAGTACCCCCCCAGGTTCTTCAAAGA AACCACTGGATGGAGAA

Oligo 5539 (SEQ ID No. 18):

GGTCGAATTCGGGCCCTCAGTCTGAGTCAGGCCCTTC

Example 5: Construction of a control plasmid carrying a sequence encoding a chimeric molecule comprising a DNA-binding domain (TetR or Cro) and the transactivator domain of the p53 protein (region 1-73).

The plasmids p53 1/73-TetR or Cro with or without tag (myc or VSV) and Hinge (Figures 7A, B and C) were obtained by cloning fragments amplified by PCR

using a plasmid carrying the cDNA for the human wild-type p53 with the aid of the oligos 5661/5662 and then digested with NcoI/NotI, NcoI/XbaI, NcoI/BamHI into the plasmids described in 3.1., predigested with NcoI/NotI, NcoI/XbaI or NcoI/BamHI.

Oligo 5661 (SEQ ID No. 19):

CAGGCCATGGAGGAGCCGCAGTCAGATCC

Oligo 5662 (SEQ ID No. 20):

CGTCGGATCCTCTAGATGCGGCCGCCACGGGGGGAGCAGCCTC TGG

Example 6: Construction of plasmids for expressing various hybrid molecules of the invention

The plasmids used for the expression of hybrid molecules were obtained by cloning fragments containing the cDNAs encoding these molecules into the NcoI/EcoRI sites of the eucaryotic expression vector pcDNA3 (Invitrogen). The various constructs thus produced are the following:

- ScFv 421: SEQ ID No. 4
- TET19: hybrid protein containing the chain ScFv421-VSV-Hinge-TetR described in Figure 6 according to Example 3.1
- TET02: hybrid protein containing the chain p53(320/393)-VSV-Hinge-TetR described in Figure 5A according to Example 4.3
- TET03: hybrid protein containing the chain p53(320/393)-Hinge-TetR described in Figure 5B according to Example 4.4

- TET04: hybrid protein containing the chain p53(320/393)-TetR described in Figure 5C according to Example 4.4

- TET07: hybrid protein containing the chain p53(1/73)-VSV-Hinge-TetR described in Figure 7A according to Example 5

Example 7: Recognition of specific double-stranded DNA sequences by the hybrid molecules of the invention

7.1. Production of the hybrid molecules

The various molecules used in this experiment were obtained by translating *in vitro* in reticulocyte lysate the molecules described in Example 6 using the TNT Coupled Reticulocyte lysate Systems kit (Promega) according to the experimental procedure described by the supplier for a total reaction volume of 50 µl.

7.2 Construction of the specific double-stranded DNA sequence

The specific double-stranded DNA sequence used in this experiment consists of two synthetic oligonucleotides whose sequence is the following:

Oligo 5997 (SEQ ID No. 24):

GATCCGACTTTCACTTTTCTCTATCACTGATAGTGAGTGGTAAACTCA

Oligo 5998 (SEQ ID No. 25):

AGCTTGAGTTTACCACTCCCTATCAGTGATAGAGAAAAGTGAAAGTCG

These two synthetic oligonucleotides were labelled with phosphorus-33 by incubating for 30 min at 37°C 10 pmol of each oligonucleotide in 10 µl of the following reaction medium:

Tris-HCl pH7.6	50 mM
$MgCl_2$	10 mM
dithiothreitol	5 mM
Spermidine	100 μM
EDTA	100 μM
[γ-^{33}P]ATP (Amersham)	50 μCi (1000-3000 Ci/mmol)
T4 kinase (Boehringer)	10 U

The two oligonucleotides thus labelled were then hybrised in the presence of 400 mM NaCl in order to reconstitute the following double-stranded sequence TetO (SEQ ID No. 26):

GATCCGACTTTCACTTTTCTCTATCACTGATAGTGAGTGGTAAACTCA

CTAGGCTCAAAGTGAAAAGAGATAGTGACTATCACTCACCATTTGAGT

7.3 Recognition of the double-stranded sequence TetO by the various hybrid molecules of the invention

The DNA-binding reaction was carried out in 50 μl of reaction medium (10 mM Tris-HCl pH7.4, 10 mM $MgCl_2$, 10 mM KCl, 6 mM β-mercaptoethanol, 0.1 mM EDTA, 0.5 mg/ml BSA) by adding the sequence TetO (10^{-10}M) prepared according to Example 7.2, 10 μl of translational product prepared according to Example 7.1 and 10^{-8}M cold competitor oligonucleotide AP2 (Promega) used to eliminate nonspecific binding. The specificity of the interaction is checked by displacing the equilibrium by adding 10 μM tetracycline (Sigma) to the reaction medium. The reaction mixtures are incubated for 15 min at 20°C and then supplemented with 10 μl of

50 % glycerol, and the final mixtures are subjected to a native 5 % polyacrylamide gel electrophoresis with migration at 200 V and 16°C. The gel is then dried and subjected to autoradiography.

The result of this experiment carried out with the hybrid molecules TET19, TET02 and TET07 is presented in Figure 9. Under these conditions, the binding of these three molecules to the specific double-stranded DNA sequence TetO is observed by a delay in the migration of the latter, and the specificity of this interaction is demonstrated by the inhibition of this delay by the addition of tetracycline.

This result therefore confirms that the hybrid molecules of the invention are capable of binding specifically to the nucleotide sequence TetO

Example 8: Specific binding of the hybrid molecules of the invention to a molecule having a transcriptional transactivating domain.

8.1. Production of the hybrid molecules of the invention and of molecules with or without a transcriptional transactivating domain.

For this experiment, the hybrid molecules of the invention ScFv 421, TET19 and TET02 according to Example 6 were produced by in vitro translation using the experimental procedure according to Example 7.1 in the presence of 44 μCi of ^{35}S-methionine (Amersham) (1175 Ci/mmol) in order to generate these radioactively

labelled hybrid molecules.

The cDNAs of the molecules with or without a transcriptional transactivating domain were cloned into the vector pBlueBacIII (Invitrogen) at the BamH1 site. Using these vectors, recombinant baculoviruses were produced and purified according to the instructions of the manufacturer. The molecules were produced by infecting, with the recombinant baculovirus, sf9 insect cells according to the manufacturer's experimental procedure. Protein extracts at the final protein concentration of 10 mg/ml are prepared according to the procedure described by K. Ory et al. (K. Ory, EMBO J., 13, 3496-3504, 1994). These molecules are the following:

- p53 (1/393): wild-type p53 protein
- p53 (1/320): wild-type p53 protein limited to its amino acid sequence 1 to 320 and therefore lacking its oligomerization domain and the domain recognized by the monoclonal antibody pAb421.

8.2. Binding of the hybrid molecules of the invention to the molecules with or without a transcriptional transactivating domain

5 µl of each of the products of translation in vitro, prepared according to Example 8.1, were incubated with 5 µl of the baculovirus extract prepared according to Example 8.1 and 2 µg of the monoclonal antibody DO1 (Oncogene Sciences) which recognises the N-terminal end of the p53 protein for 16 hours at 4°C

in 100 µl of modified RIPA buffer (K. Ory, EMBO J., 13, 3496-3504, 1994). The immunoprecipitation is carried out as described by K. Ory et al. (K. Ory, EMBO J., 13, 3496-3504, 1994). The complexes retained by the antibody are eluted by incubating for 10 min at 80°C in the presence of 30 µl of migration buffer (Laemmli U.K., Nature, 227, 680-685, 1970) and subjected to electrophoresis on a 10 % polyacrylamide gel in a denaturing medium at 200 V according to the procedure previously described (Laemmli U.K., Nature, 227, 680-685, 1970). The gel is then dried and visualized with the aid of an instantimager (Packard Instruments) which makes it possible to quantify the quantities of hybrid molecules bound to the molecule with or without a transcriptional transactivating domain. The results of this experiment are represented in Figure 10.

Under these conditions, it appears clearly that the hybrid molecules exhibiting ScFv 421 (TET19) indeed recognises the p53 molecule (1/393) in a manner equivalent to ScFv 421 alone, and the hybrid molecule exhibiting the 320/393 domain (TET02) exhibits the same properties but with a much greater p53 (1/393) retaining power. Furthermore, the absence of an observed signal during the incubation with the p53 (1/320) molecule shows that these interactions are quite specific and mediated by the C-terminal end of the p53 protein (amino acids 320 to 393) as expected.

These results therefore confirm that the

hybrid molecules of the invention are quite capable of recruiting a transcriptional transactivating domain carried by a molecule for which they are specific partners.

Example 9: Functional recruitment of a transcriptional transactivating domain by the hybrid molecules of the invention

A functional recruitment of a transcriptional transactivating domain by the hybrid molecules of the invention was evaluated in an *in vivo* transactivating system in SAOS-2 cells (human osteosarcoma) deficient for the two alleles of the p53 protein, in the tumour line H358 deficient for the two alleles of the p53 protein (Maxwell & Roth, Oncogene 8, 3421, 1993) and in the tumour line HT29 deficient for one of the two alleles of the p53 protein and having one mutated allele (H273 mutation). This system is based on the use of a reporter gene which can be assayed enzymatically and which is placed under the control of a promoter containing the nucleotide units for specific recognition by the Tet repressor. (Tet Operator).

In this test, the LUC (luciferase) reporter gene placed under the control of the Tet operator is contained in the plasmid pUHC13-3 (Gossen M. & Bujard H., Proc. Natl., Acad. Sci. USA, 89, 5547-5551, 1992).

9.1 Cell lines used and culture conditions

The cell lines used in these experiments as well as their genotype linked to the p53 protein and

the culture media used for their growth are reported in the table below:

Table: cell lines

Line	p53	Culture medium	ATCC No.
SAOS-2	-/-	DMEM medium (Gibco BRL) supplemented with 10% foetal calf serum (Gibco BRL)	HTB 85
H358	-/-	RPMI 1640 medium (Gibco BRL) supplemented with 10% foetal calf serum (Gibco BRL)	
HT29	-/H273	DMEM medium (Gibco BRL) supplemented with 10% foetal calf serum (Gibco BRL)	

9.2. Plasmid for expressing the molecules having a transcriptional transactivating domain

The molecules having a transcriptional transactivating domain used in this experiment are the wild-type (wt) p53 protein and the mutant G281 and H175 of this protein. The cDNAs encoding these three proteins were inserted at the BamHI site of the vector pcDNA3 (Invitrogen).

9.3. Intracellular expression of the hybrid molecules of the invention

The hybrid molecules of the invention are expressed in cells in culture by transient transfection

using the following procedure:

The cells (3.5×10^5) are inoculated into 6-well plates containing 2 ml of culture medium, and cultured overnight in a CO_2 (5%) incubator at 37°C. The various constructs were then transfected using lipofectAMINE (Gibco BRL) as transfection agent in the following manner: 1.5 μg of total plasmid are incubated (including 0.25 μg of the reporter plasmid) with 5 μl of lipofectAMINE for 30 min with 2 ml of serum-free culture medium (transfection mixture). During this period, the cells are rinsed twice with PBS and then incubated for 4 h at 37°C with the transfection mixture, following which the latter is aspirated and replaced with 2 ml of culture medium supplemented with 10% heat-inactivated foetal calf serum and the cells allowed to grow again for 48 h at 37°C.

9.4. Detection of the activation of transcription

The activation of transcription linked to the functional recruitment of the transcriptional transactivator is detected and quantified by measuring the luciferase activity encoded by the LUC gene using the Luciferase Assay System kit (Promega) according to the manufacturer's experimental procedure.

9.5. Functional recruitment of a transcriptional transactivating domain by the molecules of the invention

This experiment was carried out using the molecules TET02, TET03 and TET07 according to Example

6. In this experiment, the molecule TET07 serves as positive control since it has its own transcriptional transactivating domain.

The results obtained in the SAOS-2 cells and presented in Figure 11 shows that the TET07 molecule is quite capable, on its own, of activating the transcription of the LUC gene placed under the control of the Tet operator unlike the construct TET02. This is in agreement with the fact that this cell line does not contain an endogenous p53 protein which cannot therefore be recruited by the TET02 molecule. On the other hand, the introduction of the wild-type p53 protein or of its G281 mutant which do not produce any signal on their own, is capable of generating a transcriptional activity in the presence of the TET02 molecule. Such a result cannot be observed with the H175 mutant described in the literature as having a nonfunctional transcriptional transactivating domain.

This result, obtained in the SAOS-2 cell line with the TET02 molecule, was able to be reproduced in a tumour line not containing an endogenous p53 (H358 cells) either and was able to be extended to the TET03 and TET04 molecules (Figure 12).

With the aim of confirming these two results in a different cellular context, the TET02 molecule as well as the positive control TET07 were expressed in the HT29 cells which exhibit a mutant endogenous p53 protein (H273), the negative control for this

experiment consisting in transfecting the empty pcDNA3 vector. The result of this experiment, which is presented in the table below, shows that the TET02 molecule is quite capable of recruiting the transcriptional transactivating domain of the endogenous p53 protein.

Table: Transcriptional activation of the hybrid molecules of the invention in HT29 cells

pcDNA3	TET07	TET02
1	59	10

These experiments as a whole therefore show that the hybrid molecules of the invention are quite capable of binding both specific double-stranded DNA sequences and proteins exhibiting a transcriptional transactivating domain, and that these molecules are capable of conditionally inducing the expression of genes of interest.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:

(A) NAME: RHONE POULENC RORER S.A.

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(E) COUNTRY: FRANCE

(F) POSTAL CODE: 92165

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(ii) TITLE OF INVENTION: CONDITIONAL EXPRESSION SYSTEM

(iii) NUMBER OF SEQUENCES: 26

(iv) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Tape

(B) COMPUTER: IBM PC compatible

(C) OPERATING SYSTEM: PC-DOS/MS-DOS

(D) SOFTWARE: PatentIn Release #1.0, Version #1.30 (EPO)

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 19 base pairs

(B) TYPE: nucleotide

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

TCTCTATCAC TGATAGGGA 19

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 17 base pairs

(B) TYPE: nucleotide

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

TATCACCGCA AGGGATA 17

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 74 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

Lys Lys Pro Leu Asp Gly Glu Tyr Phe Thr Leu Gln Ile Arg Gly Arg
1 5 10 15
Glu Arg Phe Glu Met Phe Arg Glu Leu Asn Glu Ala Leu Glu Leu Lys
20 25 30
Asp Ala Gln Ala Gly Lys Glu Pro Gly Gly Ser Arg Ala His Ser Ser
35 40 45
His Leu Lys Ser Lys Lys Gly Gln Ser Thr Ser Arg His Lys Lys Leu
50 55 60
Met Phe Lys Thr Glu Gly Pro Asp Ser Asp
65 70

(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 768 base pairs
(B) TYPE: nucleotide
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

TTACTCGCGG CCCAGCCGGC CATGGCCCAG GTGCAGCTGC AGCAGTCTGG GGCAGAGCTT 60
GTAAGGTCAG GGGCCTCAGT CAAGTTGTCC TGCACAGCTT CTGGCTTCAA CATTAAAGAC 120
TACTATATGC ACTGGGTGAA GCAGAGGCCT GAACAGGGCC TGGAGTGGAT TGGATGGATT 180
GATCCTAAGA ATGGTGATAC TGAATATGCC CCGAAGTTCC AGGGCAAGGC CACTATGACT 240
GCAGACACAT CCTCCAATAC AGCCTACCTG CAGCTCAGCA GCCTGGCATC TGAGGACACT 300
GCCGTGTATT ATTGTAATTT TTACGGGGAT GCTTTGGACT ATTGGGGCCA AGGGACCACG 360

GTCACCGTCT CCTCAGGTGG AGGCGGTTCA GGCGGAGGTG GCTCTGGCGG TGGCGGATCG 420

GATGTTTTGA TGACCCAAAC TCCACTCACT TTGTCGGTTA CCATTGGACA ACCAGCCTCC 480

ATCTCTTGCA AGTCAAGTCA GAGCCTCTTG GATAGTGATG GAAAAACATA TTTGAATTGG 540

TTGTTACAGA GGCCAGGCCA GTCTCCAAAG CGCCTAATCT ATCTGGTGTC TAAACTGGAC 600

TCTGGAGTCC CTGACAGGTT CACTGGCAGT GGATCAGGGA CAGATTTCAC ACTTAAAATC 660

AACAGAGTGG AGGCTGAGGA TTTGGGAGTT TATTATTGCT GGCAAGGTAC ACATTCTCCG 720

CTTACGTTCG GTGCTGGCAC CAAGCTGGAA ATTAAACGGG CGGCCGCA 768

(2) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 15 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

Gly Gly Gly Gly Ser Gly Gly Gly Gly Ser Gly Gly Gly Gly Ser

1 5 10 15

(2) INFORMATION FOR SEQ ID NO: 6:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 30 base pairs

(B) TYPE: nucleotide

(C) STRANDEDNESS: double

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(ix) FEATURE:

(A) NAME/KEY: CDS

(B) LOCATION: 1..30

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

```
CCC AAG CCC AGT ACC CCC CCA GGT TCT TCA                    30
Pro Lys Pro Ser Thr Pro Pro Gly Ser Ser
 1               5                  10
```

(2) INFORMATION FOR SEQ ID NO: 7:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 18 base pairs

(B) TYPE: nucleotide

(C) STRANDEDNESS: double

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(ix) FEATURE:

(A) NAME/KEY: CDS

(B) LOCATION: 1..18

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

ATG AAC CGG CTG GGC AAG 18

Met Asn Arg Leu Gly Lys

1 5

(2) INFORMATION FOR SEQ ID NO: 8:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 33 base pairs

(B) TYPE: nucleotide

(C) STRANDEDNESS: double

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(ix) FEATURE:

(A) NAME/KEY: CDS

(B) LOCATION: 1..33

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

GAA CAA AAA CTC ATC TCA GAA GAG GAT CTG AAT 33

Glu Gln Lys Leu Ile Ser Glu Glu Asp Leu Asn

1 5 10

(2) INFORMATION FOR SEQ ID NO: 9:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 7 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

Pro Lys Lys Lys Arg Lys Val
1 5

(2) INFORMATION FOR SEQ ID NO: 10:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 66 base pairs

(B) TYPE: nucleotide

(C) STRANDEDNESS: double

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10:

GGCTCTAGAC CCAAGCCCAG TACCCCCCCA GGTTCTTCAA CGCGTGGATC CATGTCCAGA 60

TTAGATAAAA GTAAAG 66

(2) INFORMATION FOR SEQ ID NO: 11:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 51 base pairs

(B) TYPE: nucleotide

(C) STRANDEDNESS: double

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 11:

CGTACGGAAT TCGGGCCCTT ACTCGAGGGA CCCACTTTCA CATTTAAGTT G 51

(2) INFORMATION FOR SEQ ID NO: 12:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 66 base pairs

(B) TYPE: nucleotide

(C) STRANDEDNESS: double

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 12:

GGCTCTAGAC CCAAGCCCAG TACCCCCCCA GGTTCTTCAA CGCGTGGATC CATGGAACAA 60

CGCATAACCC TGAAAG 66

(2) INFORMATION FOR SEQ ID NO: 13:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 51 base pairs

(B) TYPE: nucleotide

(C) STRANDEDNESS: double

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 13:

CGTACGGAAT TCGGGCCCTT ACTCGAGTGC TGTTGTTTTT TTGTTACTCG G

(2) INFORMATION FOR SEQ ID NO: 14:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 35 base pairs

(B) TYPE: nucleotide

(C) STRANDEDNESS: double

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 14:

CAGGCCATGG CATGAAGAAA CCACTGGATG GAGAA 35

(2) INFORMATION FOR SEQ ID NO: 15:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 43 base pairs

(B) TYPE: nucleotide

(C) STRANDEDNESS: double

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 15:

CGTCGGATCC TCTAGATGCG GCCGCGTCTG AGTCAGGCCC TCC 43

(2) INFORMATION FOR SEQ ID NO: 16:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 31 base pairs

(B) TYPE: nucleotide

(C) STRANDEDNESS: double

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 16:

CAGGCTCGAG AAGAAACCAC TGGATGGAGA A 31

(2) INFORMATION FOR SEQ ID NO: 17:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 61 base pairs

(B) TYPE: nucleotide

(C) STRANDEDNESS: double

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 17:

CAGGCTCGAG CCCAAGCCCA GTACCCCCCC AGGTTCTTCA AAGAAACCAC TGGATGGAGA 60

A 61

(2) INFORMATION FOR SEQ ID NO: 18:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 37 base pairs

(B) TYPE: nucleotide

(C) STRANDEDNESS: double

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 18:

GGTCGAATTC GGGCCCTCAG TCTGAGTCAG GCCCTTC 37

(2) INFORMATION FOR SEQ ID NO: 19:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 29 base pairs

(B) TYPE: nucleotide

(C) STRANDEDNESS: double

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 19:

CAGGCCATGG AGGAGCCGCA GTCAGATCC 29

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 18:

GGTCGAATTC GGGCCCTCAG TCTGAGTCAG GCCCTTC 37

(2) INFORMATION FOR SEQ ID NO: 20:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 46 base pairs

(B) TYPE: nucleotide

(C) STRANDEDNESS: double

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 20:

CGTCGGATCC TCTAGATGCG GCCGCCACGG GGGGAGCAGC CTCTGG 46

(2) INFORMATION FOR SEQ ID NO: 21:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 66 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 21:

Met Glu Gln Arg Ile Thr Leu Lys Asp Tyr Ala Met Arg Phe Gly Gln
1 5 10 15
Thr Lys Thr Ala Lys Asp Leu Gly Val Tyr Gln Ser Ala Ile Asn Lys
20 25 30
Ala Ile His Ala Gly Arg Lys Ile Phe Leu Thr Ile Asn Ala Asp Gly
35 40 45
Ser Val Tyr Ala Glu Glu Val Lys Pro Phe Pro Ser Asn Lys Lys Thr
50 55 60
Thr Ala
65

(2) INFORMATION FOR SEQ ID NO: 22:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 23 base pairs

(B) TYPE: nucleotide

(C) STRANDEDNESS: double

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 22:

GATCCTATCA CCGCAAGGGA TAA 23

(2) INFORMATION FOR SEQ ID NO: 23:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 23 base pairs

(B) TYPE: nucleotide

(C) STRANDEDNESS: double

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 23:

GATAGTGGCG TTCCCTATTT CGA 23

(2) INFORMATION FOR SEQ ID NO: 24:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 48 base pairs

(B) TYPE: nucleotide

(C) STRANDEDNESS: double

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 24:

GATCCGACTT TCACTTTTCT CTATCACTGA TAGTGAGTGG TAAACTCA 48

(2) INFORMATION FOR SEQ ID NO: 25:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 48 base pairs
(B) TYPE: nucleotide
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 25:

AGCTTGAGTT TACCACTCCC TATCAGTGAT AGAGAAAAGT GAAAGTCG 48

(2) INFORMATION FOR SEQ ID NO: 26:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 96 base pairs
(B) TYPE: nucleotide

(C) STRANDEDNESS: double

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 26:

GATCCGACTT TCACTTTTCT CTATCACTGA TAGTGAGTGG TAAACTCACT AGGCTCAAAG 60

TGAAAAGAGA TAGTGACTAT CACTCACCAT TTGAGT 96

CLAIMS

1. Bispecific chimeric molecule comprising a domain capable of binding selectively a defined DNA sequence and a detecting domain capable of binding specifically a transactivator or a transrepressor or a transactivating or transrepressing complex characteristic of a physiological or physiopathological state.

2. Molecule according to claim 1, characterized in that the domain capable of binding selectively a defined DNA sequence is derived from a protein capable of interacting with the DNA.

3. Molecule according to claim 2, characterized in that the domain capable of binding selectively a defined DNA sequence is derived from a eukaryotic protein.

4. Molecule according to claim 3, characterized in that the domain capable of binding selectively a defined DNA sequence is derived from the proteins p53, STAT or NFkB.

5. Molecule according to claim 2, characterized in that the domain capable of binding selectively a defined DNA sequence is derived from a prokaryotic protein.

6. Molecule according to claim 5, characterized in that the prokaryotic protein is a bacterial repressor.

7. Molecule according to claim 6,

characterized in that the domain capable of binding selectively a defined DNA sequence is derived from the tetR protein.

8. Molecule according to claim 6, characterized in that the domain capable of binding selectively a defined DNA sequence is derived from the Cro protein.

9. Molecule according to one of claims 2 to 8, characterized in that the domain capable of binding selectively a defined DNA sequence comprises the domain for interacting with DNA of the said protein.

10. Molecule according to one of claims 2 to 8, characterized in that the domain capable of binding selectively a defined DNA sequence consists of a complete protein.

11. Molecule according to claim 10, characterized in that the domain capable of binding selectively a defined DNA sequence consists of the tetR protein.

12. Molecule according to claim 10, characterized in that the domain capable of binding selectively a defined DNA sequence consists of the Cro protein.

13. Molecule according to claim 1, characterized in that the domain capable of binding specifically the transactivator or transrepressor or the transactivating or transrepressing complex is an oligomerizing domain.

14. Molecule according to claim 13, characterized in that the oligomerizing domain is a leucine zipper, an SH3 or SH2 domain.

15. Molecule according to claim 13, characterized in that the oligomerizing domain capable of binding specifically the transactivator consists of the C-terminal part of the p53 protein.

16. Molecule according to claim 15, characterized in that the oligomerizing domain consists of the C-terminal part of the p53 protein ranging from residues 320 to 393 (SEQ ID No. 3), 302-360 or 302-390.

17. Molecule according to claim 1, characterized in that the domain capable of binding specifically the transactivator or transrepressor or the transactivating or transrepressing complex is a synthetic or natural domain known to interact with the said transactivator or transrepressor or transactivating or transrepressing complex.

18. Molecule according to claim 1, characterized in that the domain capable of binding specifically the transactivator or transrepressor or the transactivating or transrepressing complex is an antibody or an antibody fragment or derivative directed against the transactivator or transrepressor or transactivating or transrepressing complex.

19. Molecule according to claim 18, characterized in that the domain capable of binding specifically the transactivator or the transactivating

complex consists of a Fab or F(ab)'2 fragment of antibodies or a VH or VL region of an antibody.

20. Molecule according to claim 18, characterized in that the domain capable of binding specifically the transactivator or the transactivating complex consists of a single-chain antibody (ScFv) comprising a VH region linked to a VL region by an arm.

21. Molecule according to claim 1, characterized in that the DNA-binding domain and the transactivator-binding domain are linked to each other through an arm.

22. Molecule according to claim 21, characterized in that the arm consists of a peptide comprising 5 to 30 amino acids and, preferably, 5 to 20 amino acids.

23. Molecule according to claim 22, characterized in that the arm is chosen from the peptides of sequence SEQ ID No. 5 or SEQ ID No. 6.

24. Molecule according to one of the preceding claims, characterized in that the DNA-binding domain is situated at the N-terminal position and the transactivator-binding domain is situated at the C-terminal position.

25. Molecule according to one of claims 1 to 23, characterized in that the DNA-binding domain is situated at the C-terminal position and the transactivator-binding domain is situated at the N-terminal position.

26. Bispecific chimeric molecule of structure ScFv-VSV/myc-Hinge-TET or Cro (Figure 5A).

27. Bispecific chimeric molecule of structure ScFv-Hinge-TET or Cro (Figure 5B).

28. Bispecific chimeric molecule of structure ScFv-TET or Cro (Figure 5C).

29. Bispecific chimeric molecule of structure TET or Cro-ScFv (Figure 5D).

30. Bispecific chimeric molecule of structure TET or Cro-Hinge-ScFv (Figure 5E).

31. Bispecific chimeric molecule of structure Oligom-VSV/myc-Hinge-TET or Cro (Figure 5A), Oligom-Hinge-TET or Cro (Figure 5B) or Oligom-TET or Cro (Figure 5C).

32. Nucleic acid sequence encoding a chimeric molecule according to one of claims 1 to 31.

33. Nucleic acid sequence according to claim 32, characterized in that it is a DNA sequence.

34. Nucleic acid sequence according to claim 32 or 33, characterized in that it is part of a vector.

35. Conditional system for the expression of genes comprising:

- a chimeric molecule as defined in claims 1 to 31, and

- an expression cassette comprising a regulatory sequence, a minimal transcriptional promoter and the said gene.

36. Conditional system according to claim

35, characterized in that the DNA-binding domain of the chimeric molecule is represented by all or part of TetR and the regulatory sequence comprises the sequence SEQ ID No. 1 or a derivative thereof, optionally repeated several times.

37. Conditional system according to claim 35, characterized in that the DNA-binding domain of the chimeric molecule is represented by all or part of Cro and the regulatory sequence comprises the sequence SEQ ID No. 2 or a derivative thereof, optionally repeated several times.

38. Conditional system according to one of claims 35 to 37, characterized in that the minimal promoter comprises an INR or TATA box.

39. Conditional system according to claim 38, characterized in that the minimal promoter is derived from the promoter of the thymidine kinase gene.

40. Conditional system according to claim 39, characterized in that the minimal promoter is composed of nucleotides -37 to +19 of the promoter of the thymidine kinase gene.

41. Vector comprising:

- a nucleic acid sequence encoding a chimeric molecule according to one of claims 1 to 31, and
- an expression cassette comprising a regulatory sequence, a minimal transcriptional promoter and a coding sequence of interest.

42. Vector according to claim 41,

characterized in that the minimal transcriptional promoter is defined according to claims 38 to 40.

43. Vector according to claim 41, characterized in that the DNA-binding domain of the chimeric molecule is represented by all or part of TetR and the regulatory sequence comprises the sequence SEQ ID No. 1 or a derivative thereof, optionally repeated several times.

44. Vector according to claim 41, characterized in that the DNA-binding domain of the chimeric molecule is represented by all or part of Cro and the regulatory sequence comprises the sequence SEQ ID No. 2 or a derivative thereof, optionally repeated several times.

45. Vector according to one of claims 41 to 44, characterized in that the coding sequence of interest is a DNA sequence encoding a therapeutic product.

46. Vector according to claim 45, characterized in that the therapeutic product is a toxic polypeptide or peptide.

47. Vector according to claim 46, characterized in that the toxic therapeutic product is chosen from diphtheria toxin, pseudomonas toxin, ricin A, thymidine kinase, cytosine deaminase, protein Grb3-3, or ScFv Y28.

48. Vector according to one of claims 41 to 47, characterized in that it is a plasmid vector.

49. Vector according to one of claims 41 to 47, characterized in that it is a viral vector.

50. Vector according to claim 49, characterized in that it is a defective recombinant adenovirus.

51. Vector according to claim 49, characterized in that it is a defective recombinant retrovirus.

52. Pharmaceutical composition comprising at least one vector according to one of claims 41 to 51.

53. Nucleic acid comprising the sequence SEQ ID No. 4.

54. Molecule according to claim 1, characterized in that the transactivator characteristic of a physiological or physiopathological state is a protein of viral, parasitic, mycobacterial or cellular origin having a transcriptional transactivating activity.

55. Molecule according to claim 54, characterized in that the transactivator is a viral protein chosen from the HIV virus Tat protein, the papilloma virus E6/E7 proteins and the Epstein-Barr virus EBNA protein.

56. Molecule according to claim 54, characterized in that the transactivator is a cellular protein, preferably the mutated or wild-type p53 protein.

57. Molecule according to claim 1,

characterized in that the transactivator or transactivating complex characteristic of a physiological or physiopathological state is a protein appearing in an infected or hyperproliferative cell.

Abstract

A novel conditional gene expression system particularly using the creation and expression of bispecific chimeric molecules including a domain capable of selectively binding a given DNA sequence and a sensing domain capable of specifically binding a transactivator or transrepressor or a transactivator or transrepressor complex.

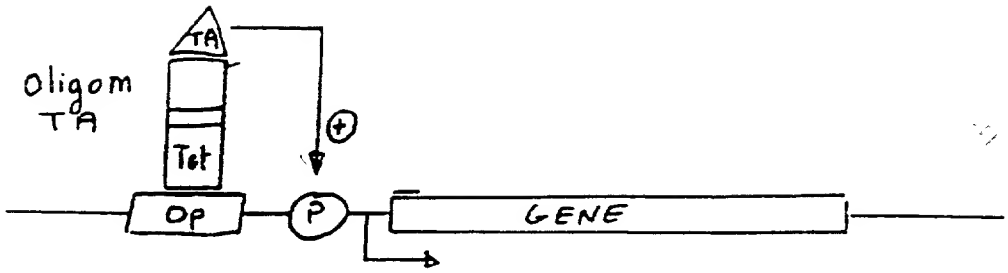


Figure 1A

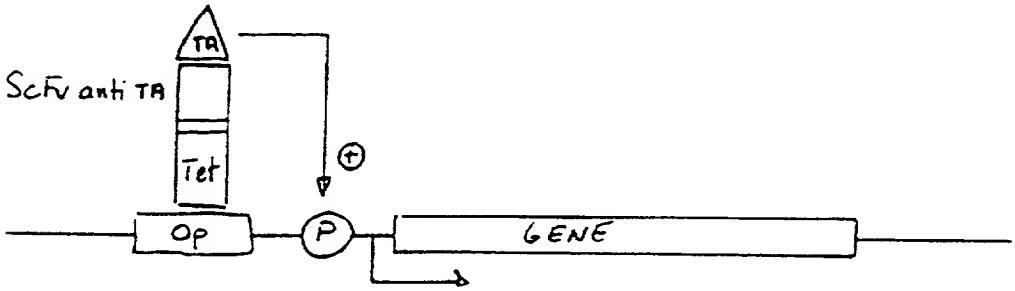


Figure 1B

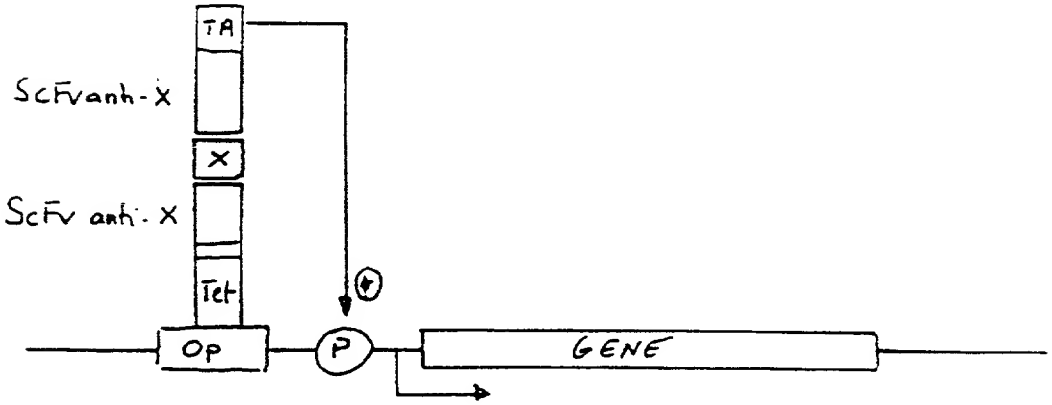
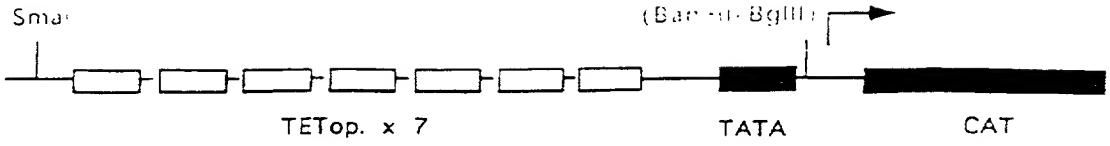


Figure 2



TETop. = GACTTTCACTTTTCTCTATCACTGATAGGGAGTGGTAAACTC

Figure 3

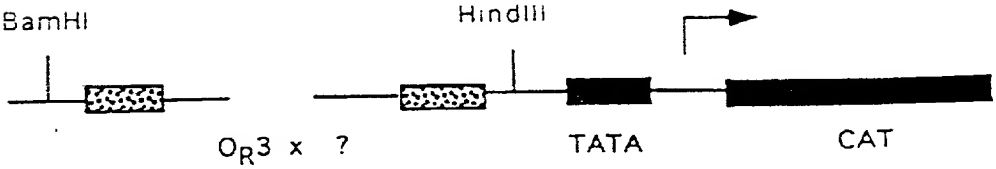


Figure 4

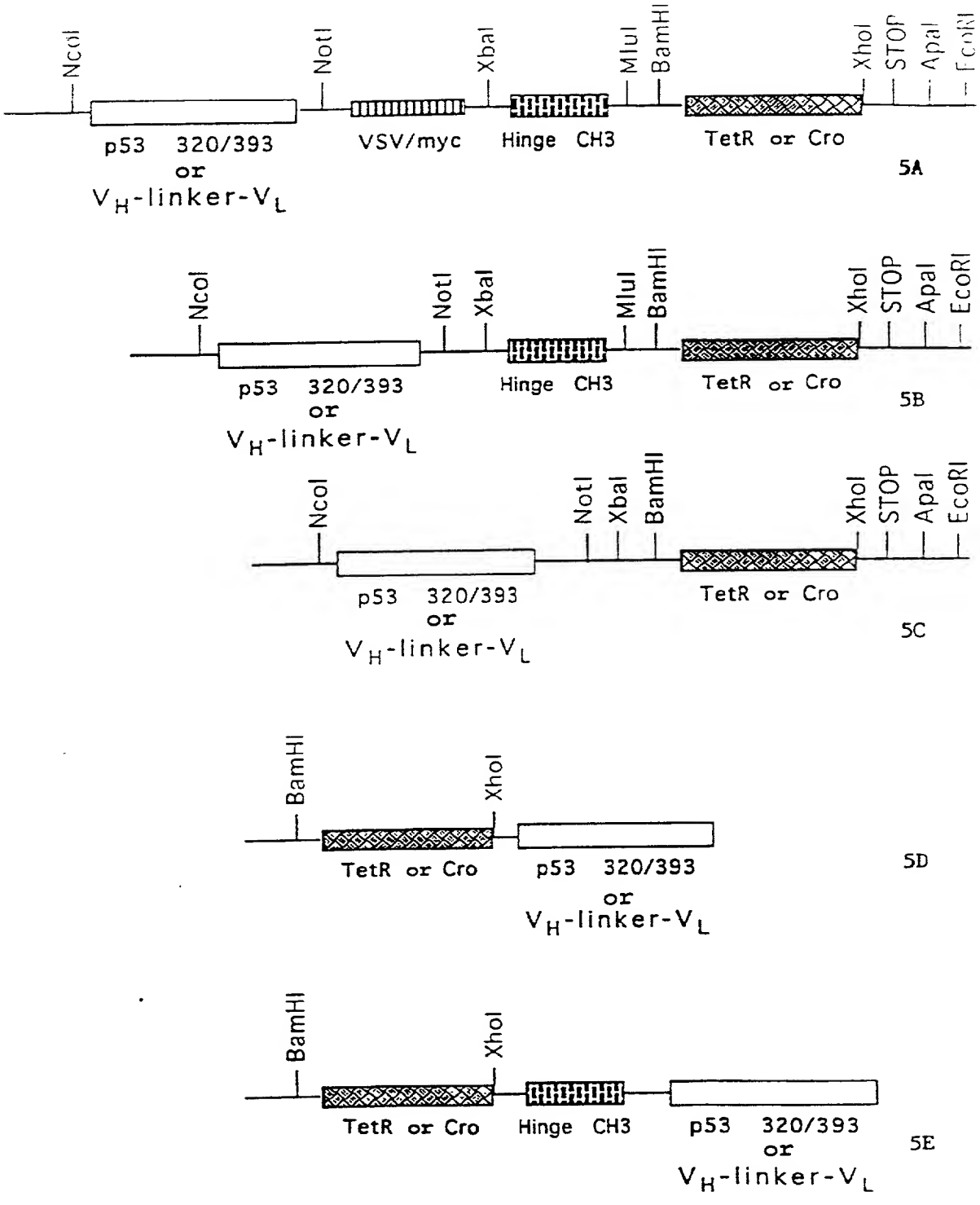
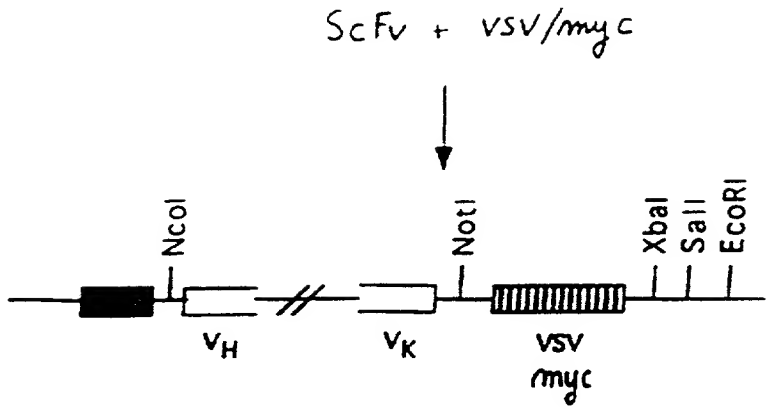

NcoI
NotI
XbaI
MluI
BamHI
XhoI
STOP
ApaI
EcoRI
p53 320/393
or
V_H-linker-V_L
VSV/myc
Hinge CH3
TetR or Cro
5A
5B
5C
5D
5E

Figure 5



XbaI — Hinge CH3 — MluI — BamHI

(5474) GGC TCT AGA CCC AAG CCC AGT ACC CCC CCA GGT TCT TCA ACG CGT GGA TCC ATG TCC AGA TTA GAT AAA AGT AAA G
ATG TCT AGA TTA GAT AAA AGT AAA G
TET Rep.

TET Rep. — XhoI — ApaI — EcoRI

... CAA CTT AAA TGT GAA AGT GGG TCC CTC GAG TAA GGG CCC GAA TTC CGT ACG

(5475) CGT ACG GAA TTC GGG CCC TTA CTC GAG GGA CCC ACT TTC ACA TTT AAG TTG

XbaI — Hinge CH3 — MluI — BamHI

(5531) GGC TCT AGA CCC AAG CCC AGT ACC CCC CCA GGT TCT TCA ACG CGT GGA TCC ATG GAA CAA CGC ATA ACC CTG AAA G
ATG GAA CAA CGC ATA ACC CTG AAA G
CRO

CRO — XhoI — ApaI — EcoRI

. CCG AGT AAC AAA AAA ACA ACA GCA CTC GAG TAA GGG CCC GAA TTC CGT ACG

(5532) CGT ACG GAA TTC GGG CCC TTA CTC GAG TGC TGT TGT TTT TTT GTT ACT CGG

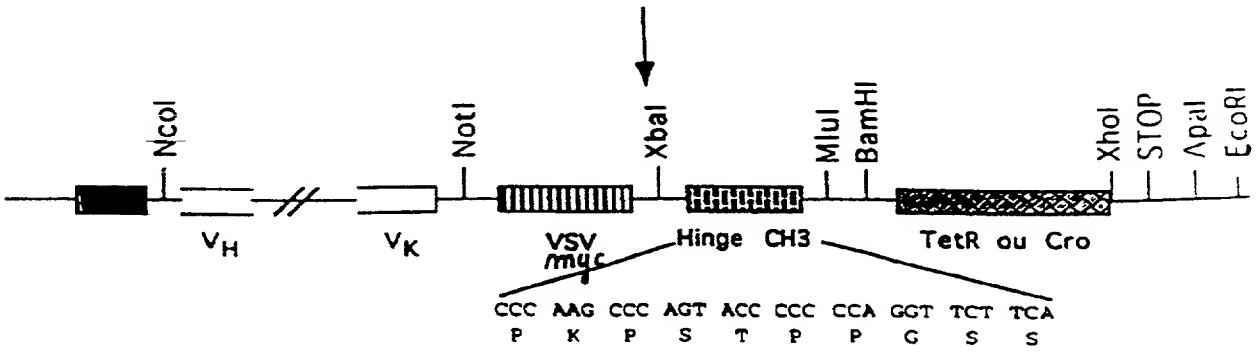


Figure 6

REPLACEMENT SHEET (RULE 26)

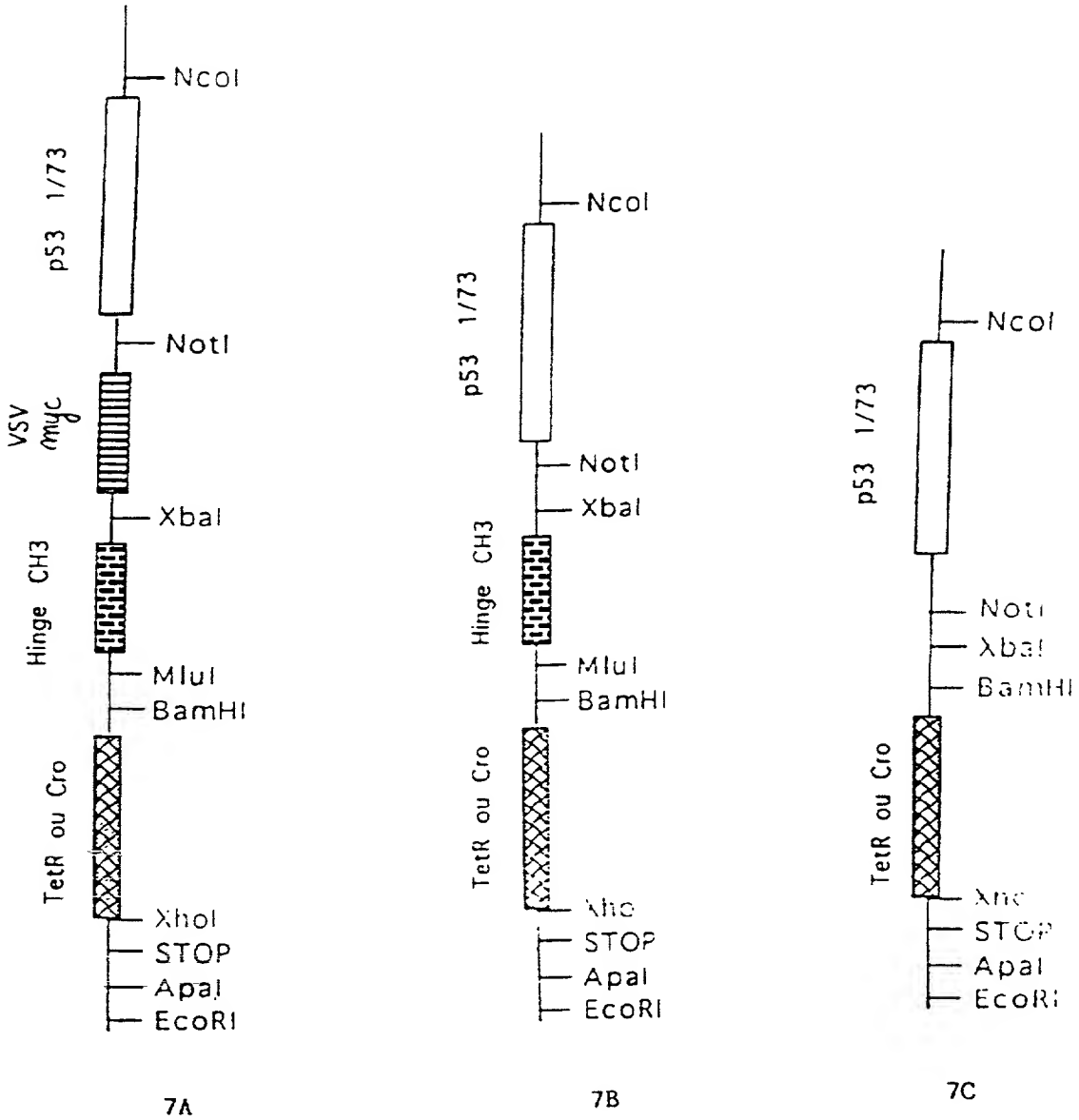
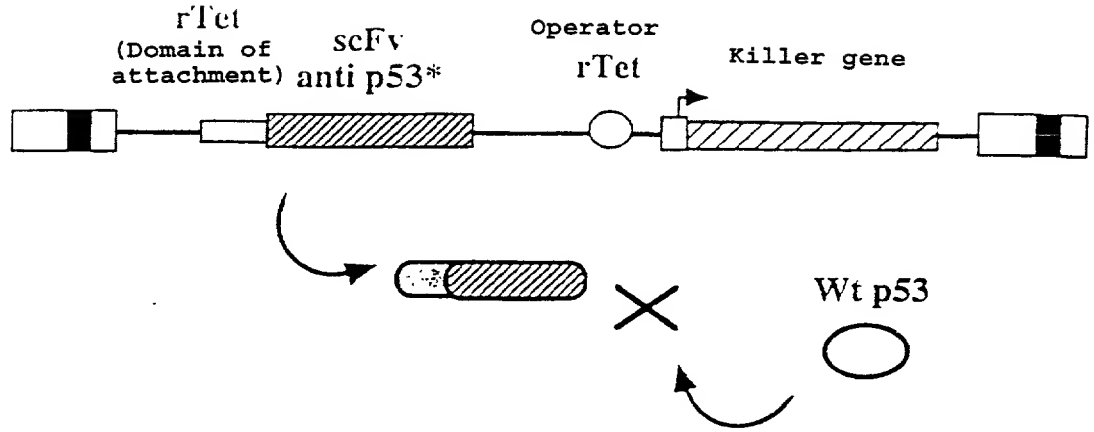
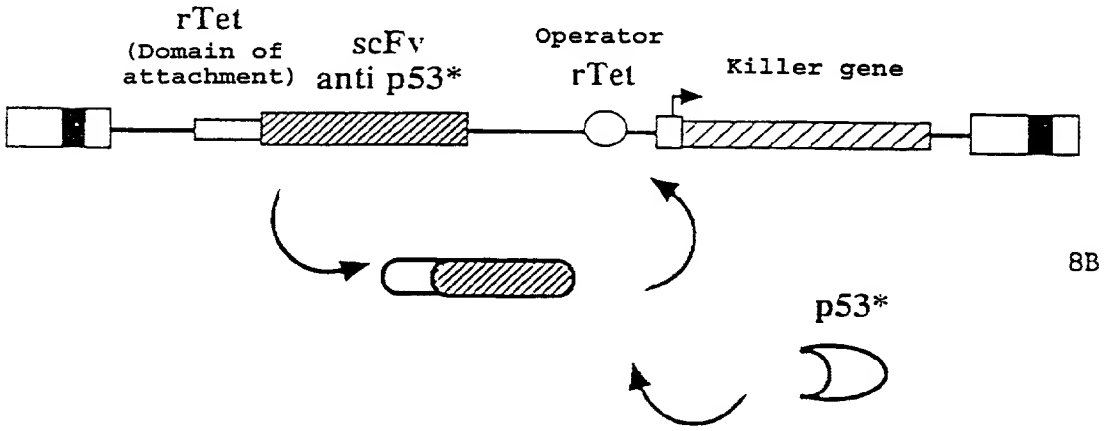


Figure 7

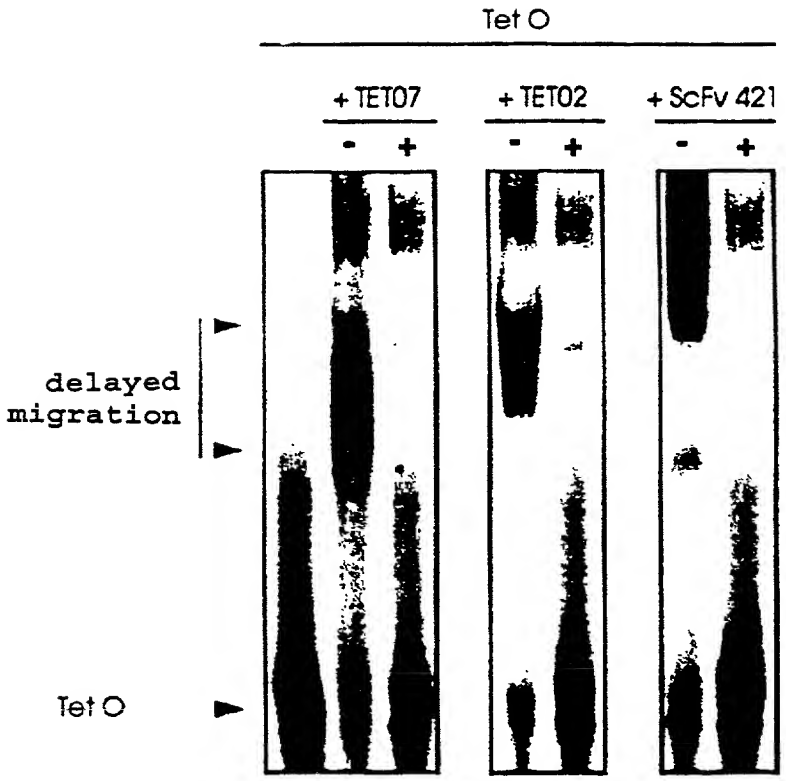


8A



8B

Figure 8



-: without tetracycline
+: with tetracycline

Figure 9

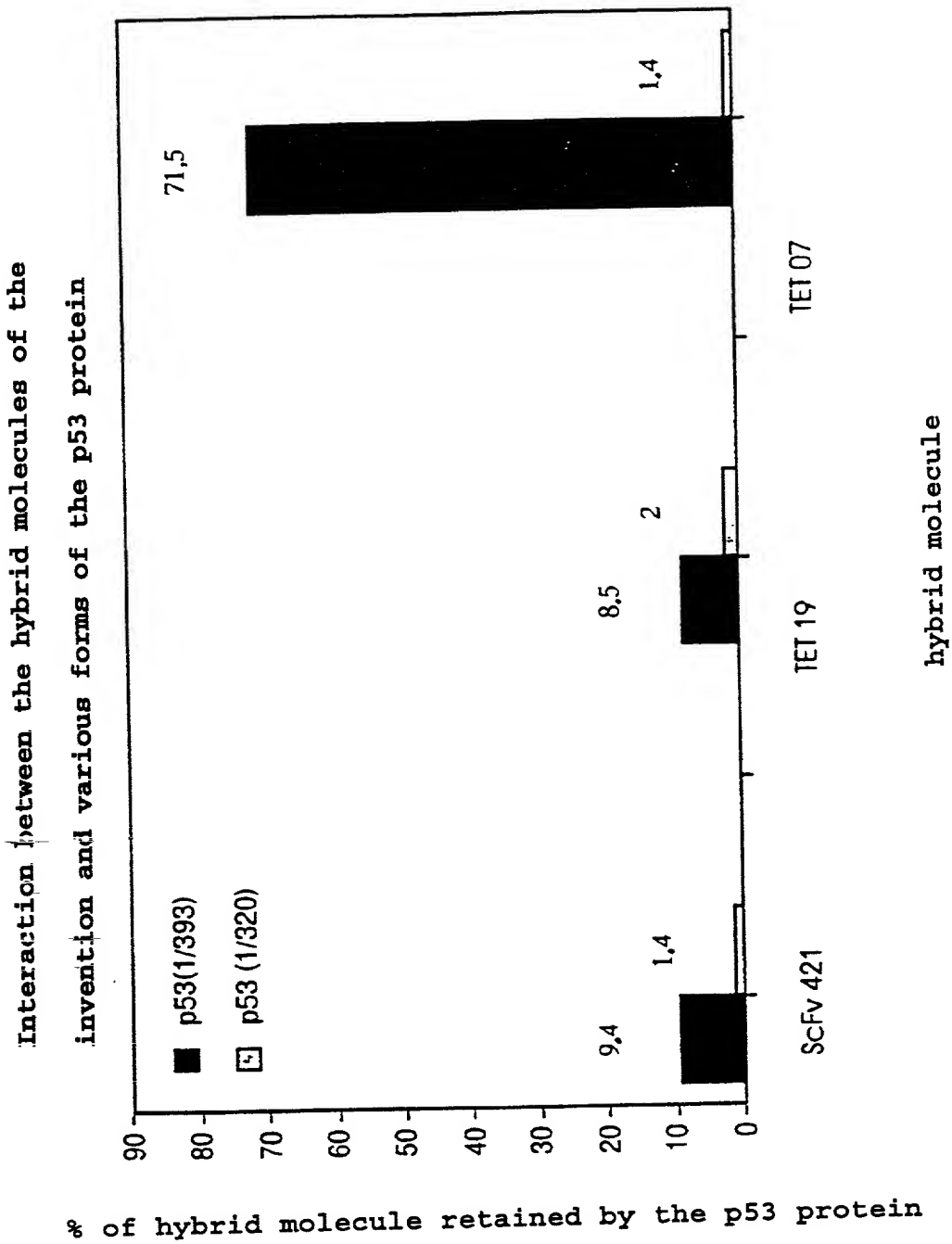

Interaction between the hybrid molecules of the invention and various forms of the p53 protein
p53(1/393)
p53 (1/320)
71,5
1,4
8,5
2
9,4
1,4
TEI 07
TEI 19
ScFv 421
hybrid molecule
90
80
70
60
50
40
30
20
10
0
% of hybrid molecule retained by the p53 protein

Figure 10

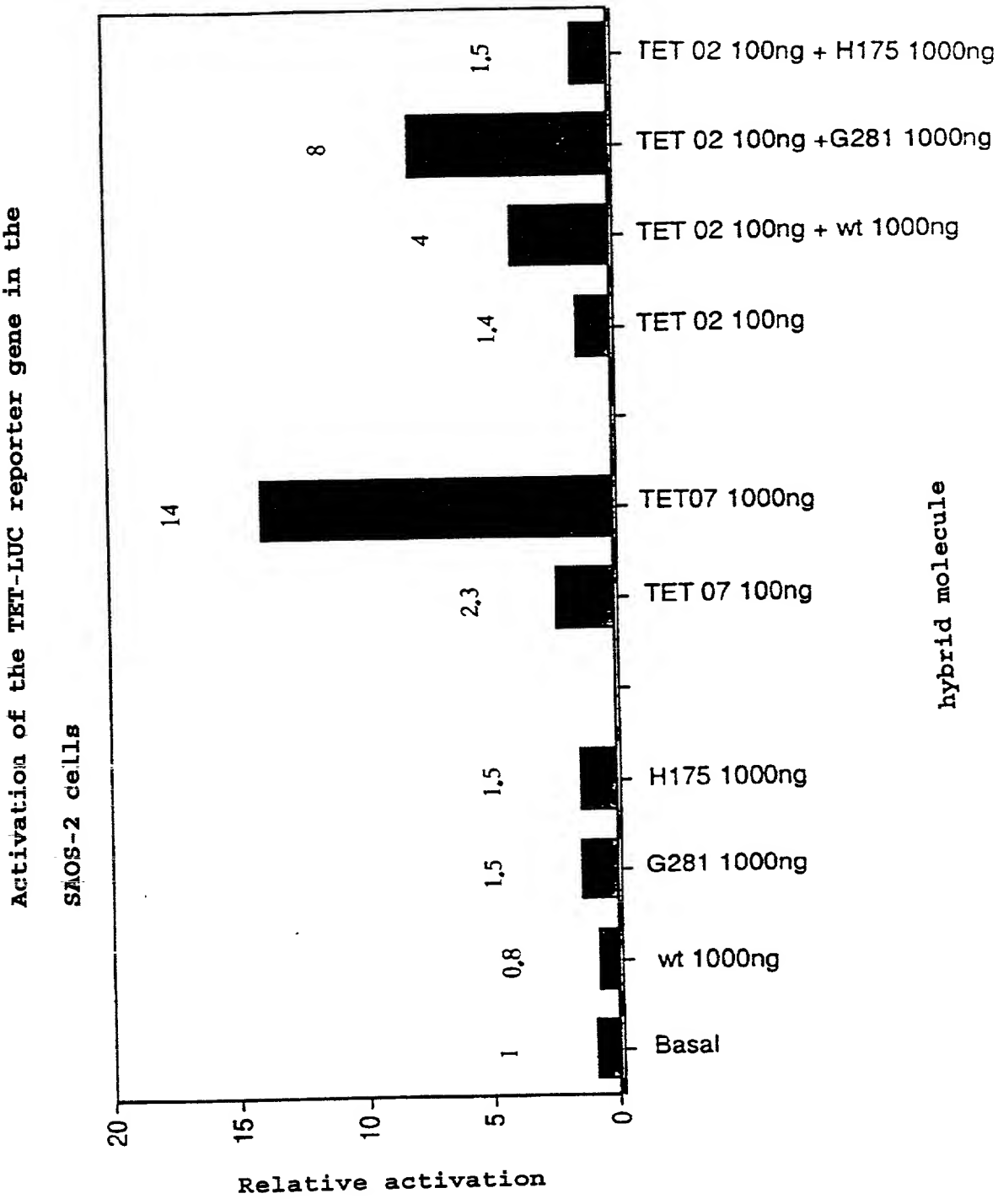

Activation of the TET-LUC reporter gene in the SAOS-2 cells
hybrid molecule
Relative activation
0
5
10
15
20
Basal
wt 1000ng
G281 1000ng
H175 1000ng
TET 07 100ng
TET07 1000ng
TET 02 100ng
TET 02 100ng + wt 1000ng
TET 02 100ng +G281 1000ng
TET 02 100ng + H175 1000ng
1
0,8
1,5
1,5
2,3
14
1,4
4
8
1,5

Figure 11

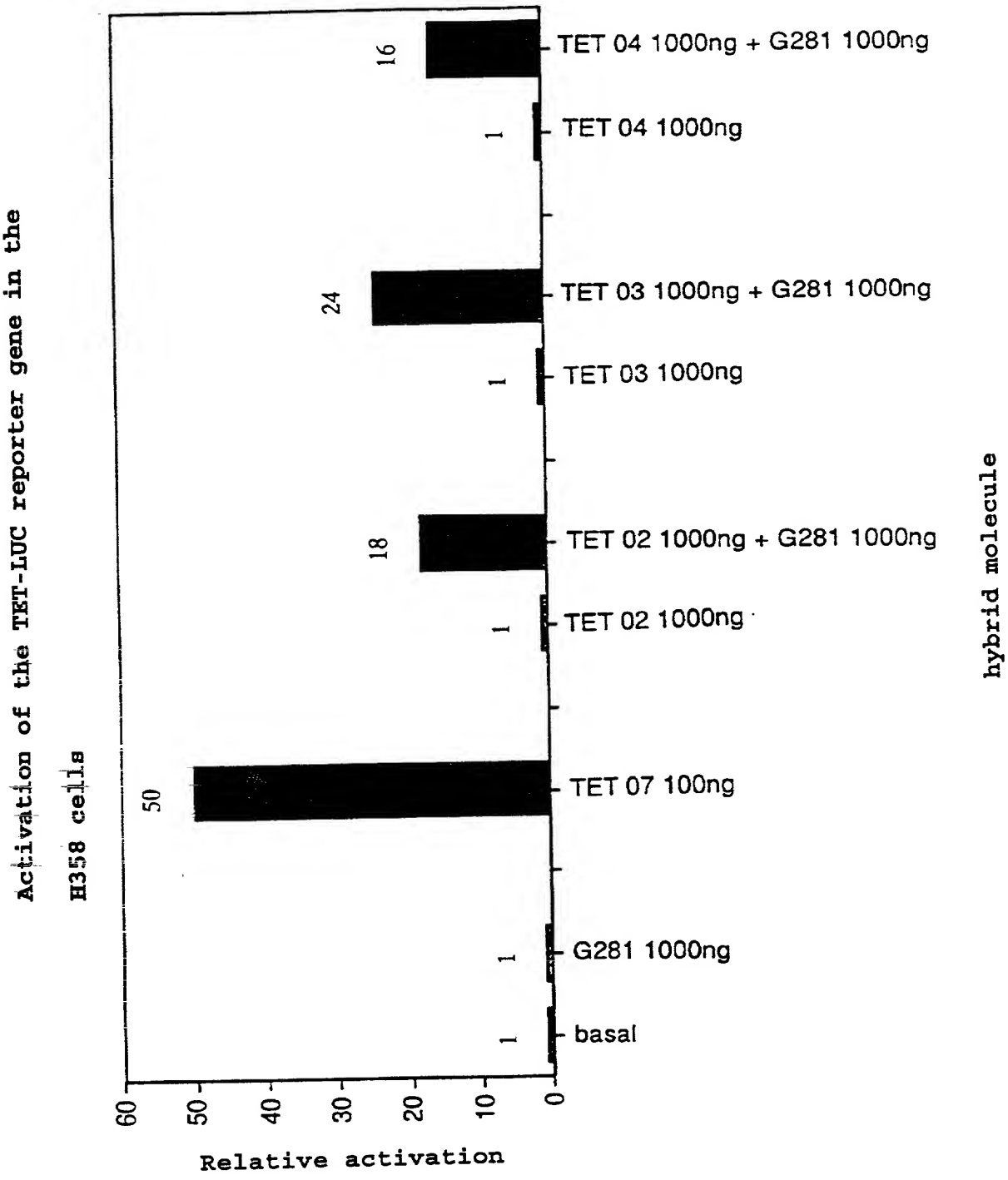

Activation of the TET-LUC reporter gene in the
H358 cells
hybrid molecule
TET 04 1000ng + G281 1000ng
TET 04 1000ng
TET 03 1000ng + G281 1000ng
TET 03 1000ng
TET 02 1000ng + G281 1000ng
TET 02 1000ng
TET 07 100ng
G281 1000ng
basal
16
1
24
1
18
1
50
1
1
0
10
20
30
40
50
60
Relative activation

Figure 12

DECLARATION AND POWER OF ATTORNEY
FOR PATENT APPLICATION

As a below named inventor, I hereby declare that my residence, post office address and citizenship are as stated below next to my name; I believe that I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled

CONDITIONAL EXPRESSION SYSTEM

the international specification of which was filed on March 29, 1996 as Application Serial No. PCT/FR96/00477 which notice of transmission was given on October 3, 1996, by the International Bureau. I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment(s) referred to above. I acknowledge the duty to disclose information which is material to the examination of this application in accordance with Title 37, Code of Federal Regulations, Section 1.56(a). I hereby claim foreign priority benefits under Title 35, United States Code, Section 119 of a foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed.

Prior Foreign Applications(s)			Priority Claimed	
FR95/03841	France	31 March 1995	X	
(Number)	(Country)	(Day/Month/Year Filed)	Yes	No
(Number)	(Country)	(Day/Month/Year Filed)	Yes	No
(Number)	(Country)	(Day/Month/Year Filed)	Yes	No

I hereby claim the benefit under Title 35, United States Code, Section 120 of any United States application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of Title 35, United States Code, Section 112, I acknowledge the duty to disclose material information as defined in Title 37, Code of Federal Regulations, Section 1.56(a) which occurred between the filing date of the prior application and the national or PCT International filing date of this application:

(Application Serial No.)	(Filing Date)	(Status-Patented, Pending or Abandoned)
(Application Serial No.)	(Filing Date)	(Status-Patented, Pending or Abandoned)

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

POWER OF ATTORNEY: I (We) hereby appoint the attorneys associated with the Customer Number provided below as my (our) attorneys, with full powers of substitution and revocation, to prosecute this application and transact all business in the Patent and Trademark Office connected therewith:

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100

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December 9th, 1997 Date	Signature

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200

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Date Dec 9 Signature B Tocque

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